IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Shimon Weiss	Art Unit: 2877
Appl. No.: 10/561,448	Examiner: F.L. Evans
Confirmation No.: 8178	
Filed: December 20, 2005	Atty. Docket No.: 58086-226455
For: MODULATED EXCITATION FLUORESCENCE ANALYSIS	Customer No. 26694 PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 C.F.R. § 1.131

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, being duly warned, declare the following:

1. I am a co-inventor of the subject matter described and claimed in the above-identified U.S. patent application. I have reviewed the claims of this application as currently amended.

2. I understand that the Office Action dated November 30, 2007 rejected the examined claims of this patent application under 35 U.S.C. § 102(a) over published German patent application Publication No. DE 10210737 A1 by Krieger et al. that published March 20, 2003.

3. I, together with my co-inventors, conceived the invention described and claimed in at least independent claims 1 and 21 of this application, and reduced it to practice, prior to the March 20, 2003 publication date of the cited reference. Our prior invention is evidenced by a copy of a presentation by one of the co-inventors, Achillefs Kapanidis, at the Single-Molecule Biophysics Conference in Aspen, CO on January 7, 2003, (copy attached as Exhibit A).

4. As documented by Exhibit A, my co-inventors and I conceived the invention of at least current independent claims 1 and 21, and reduced it to practice, prior to January 7, 2003.

5. The acts described above in paragraphs 3 and 4 were carried out in the United States of America, or else in a WTO member country.

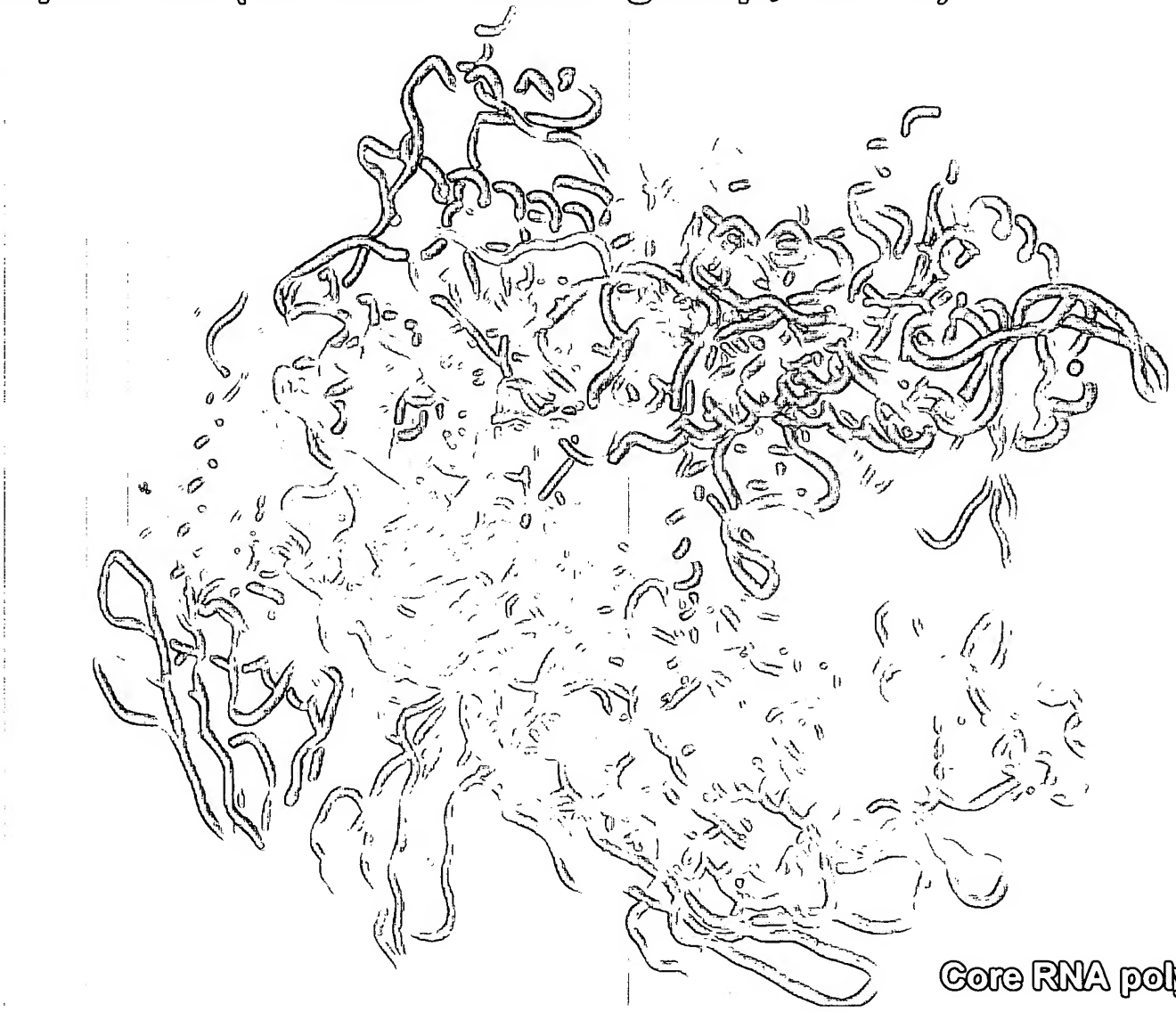
6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date	Shimon Weiss
Date	Achillefs Kapanidis
Date	Ted A. Laurence
5/27/08 Date	Nam Ki Lee Nam K. Lee

Exhibit A

Molecular Machines at Work:
Single-Molecule Analysis of Transcription by RNA Polymerase

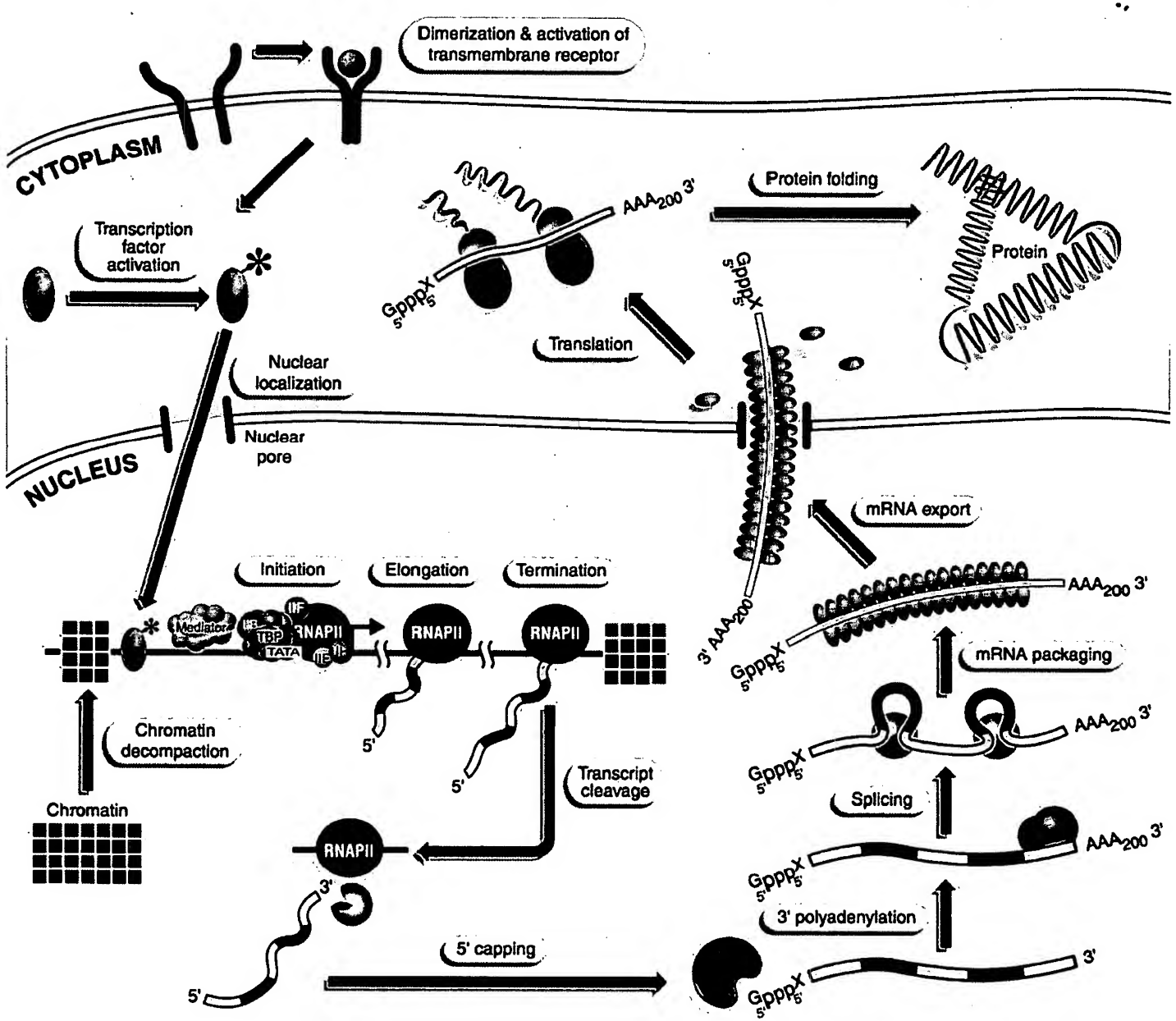
Achillefs Kapanidis (Shimon Weiss' group, UCLA)



Core RNA polymerase (Darst lab)

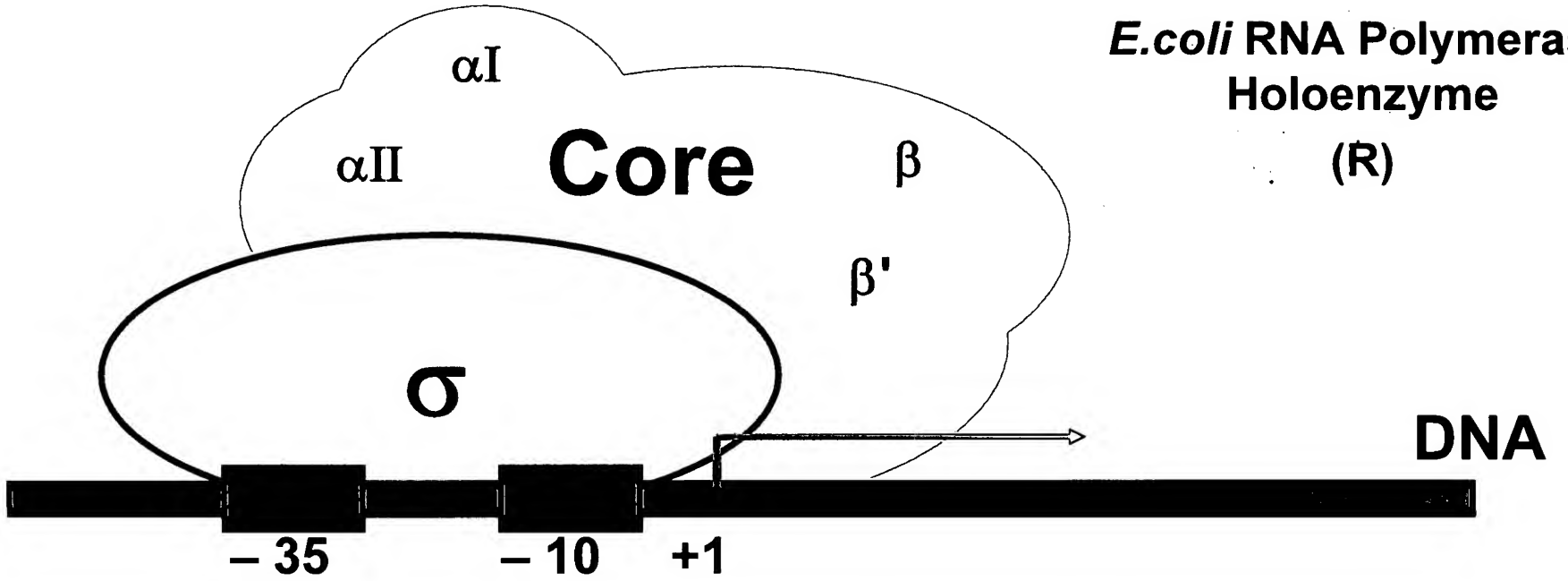
Single-Molecule Biophysics Conference: Aspen, Jan.7, 2003

GENE EXPRESSION:
The path from gene to protein

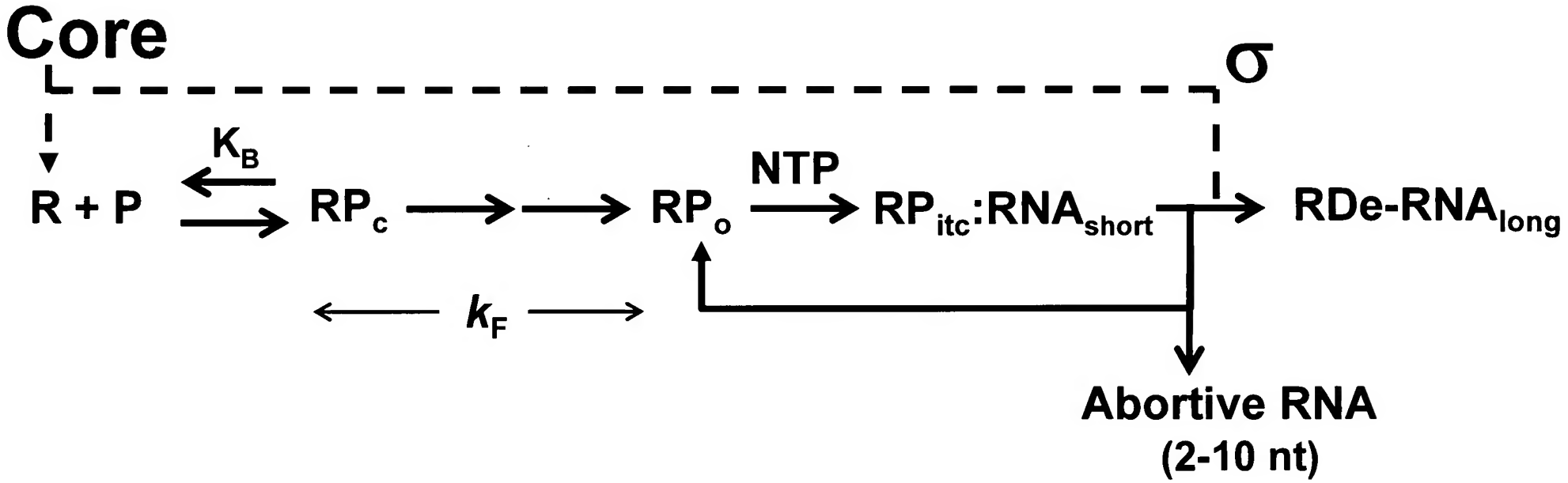


Orphanides and Reinberg, 2002

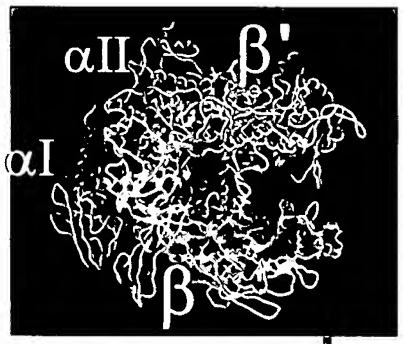
TRANSCRIPTION INITIATION



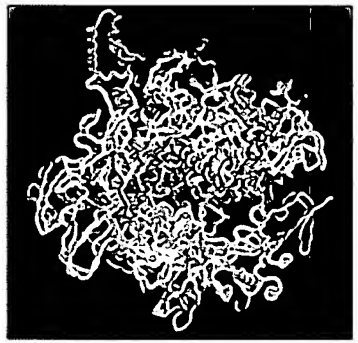
E.coli RNA Polymerase Holoenzyme (R)

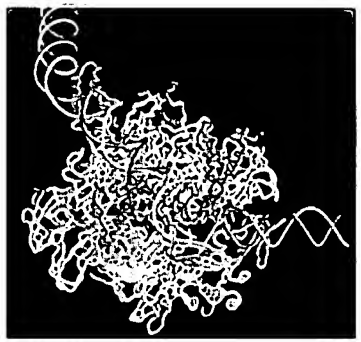


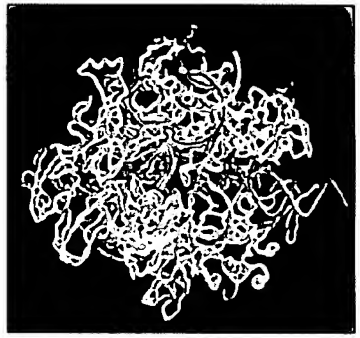
STRUCTURAL ASPECTS OF TRANSCRIPTION



RNAP core ($\alpha_2\beta\beta'$)


RNAP holoenzyme (core + σ)


RP_o


RD_e (model)

X-ray structures → static snapshots of the machine

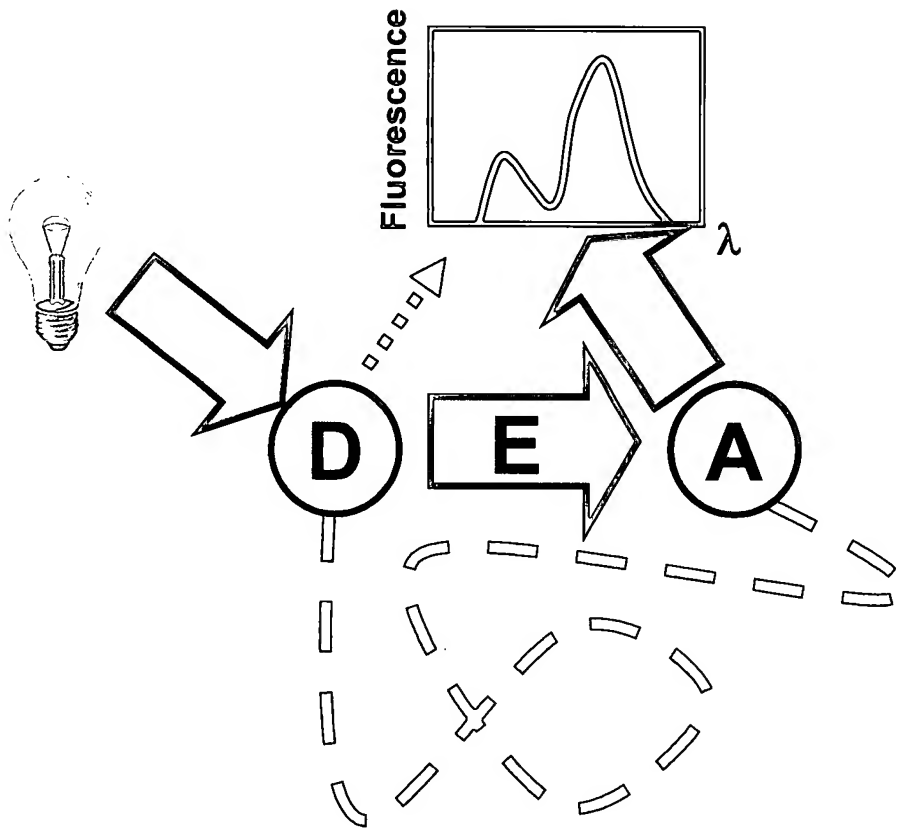
SMD: "movie" of the dynamic process

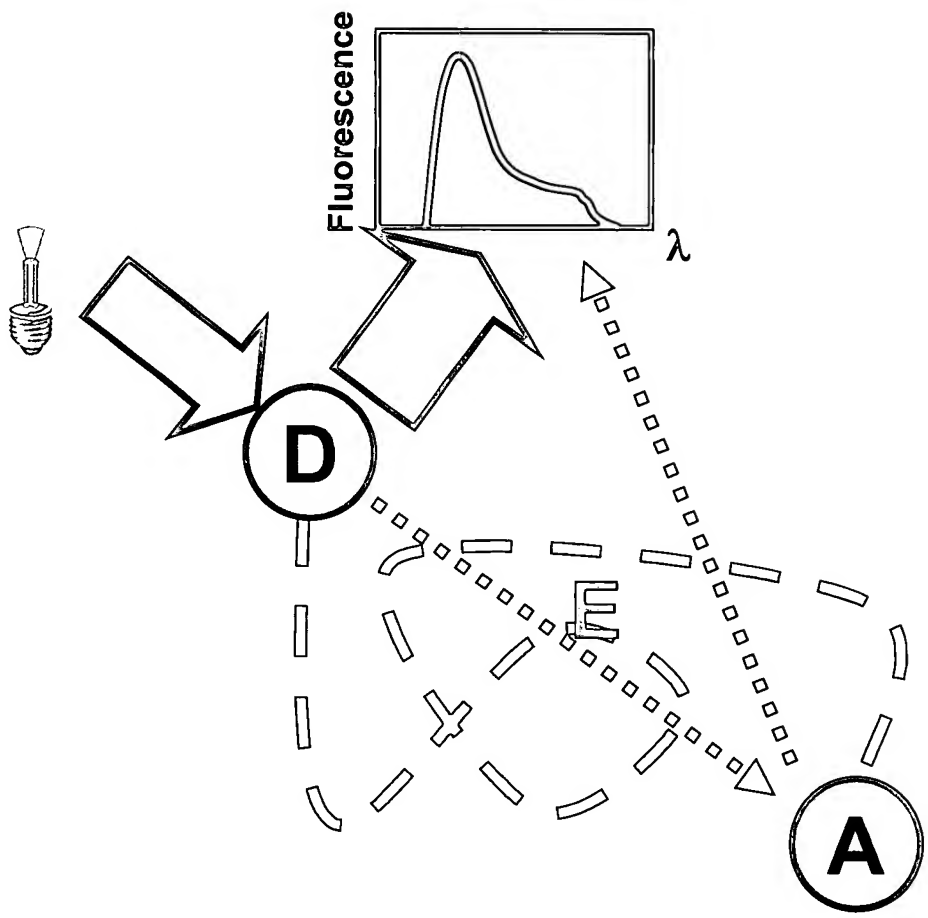
Structure
Dynamics
Local Environment
→ Intermediates
Kinetics
→ Timing
of Events
→ MECHANISM

Young *et al.*, 2002

FÖRSTER RESONANCE ENERGY TRANSFER (FRET):

A "MOLECULAR RULER" FOR THE 2-10 nm REGIME





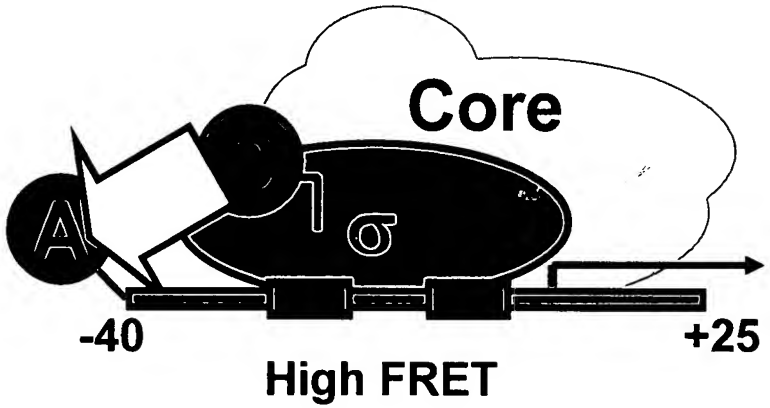
$$\text{FRET Efficiency, } E = [1 + (R/R_o)^6]^{-1}$$

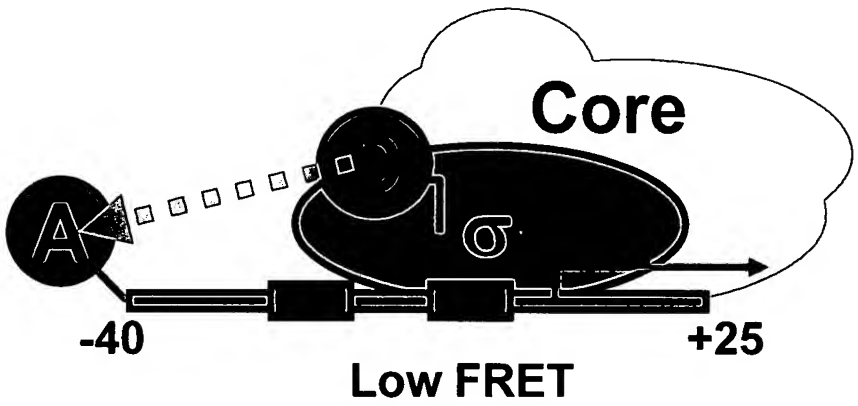
R = D-A Distance

TRAILING-EDGE and LEADING-EDGE FRET:

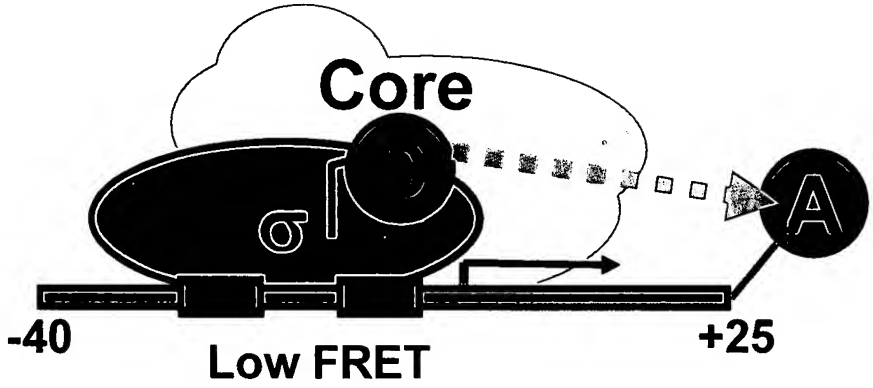
Assay of translocation of a protein relative to a nucleic acid

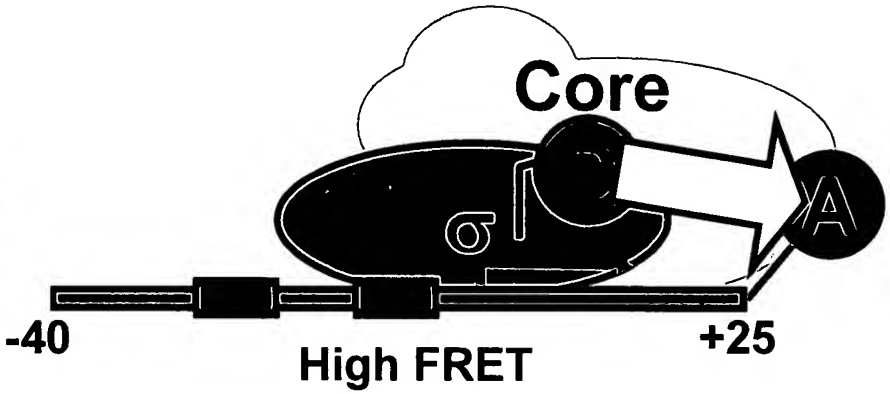
Trailing-edge FRET





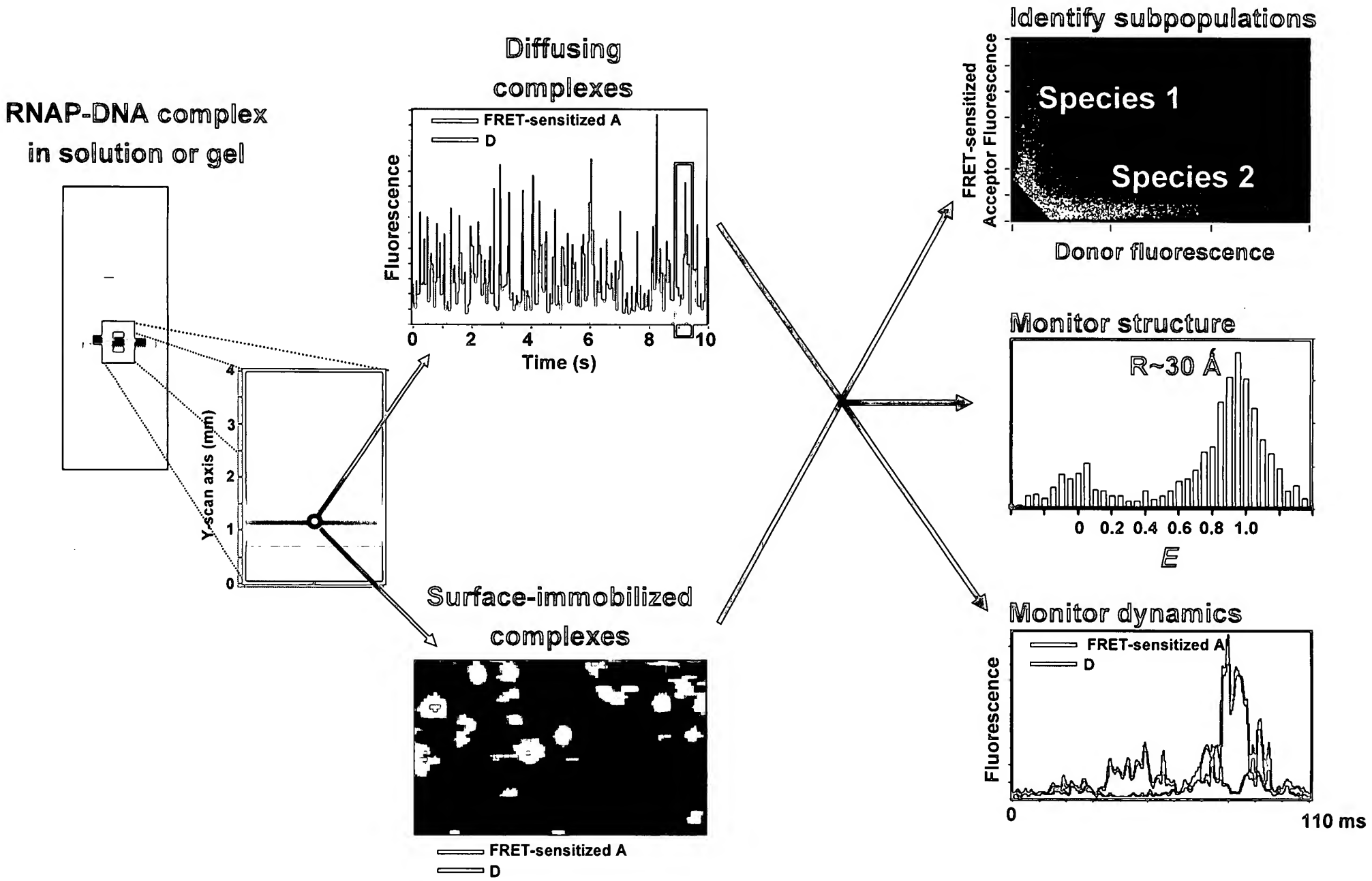
Leading-edge FRET





Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

sp-FRET ON RNAP-DNA COMPLEXES

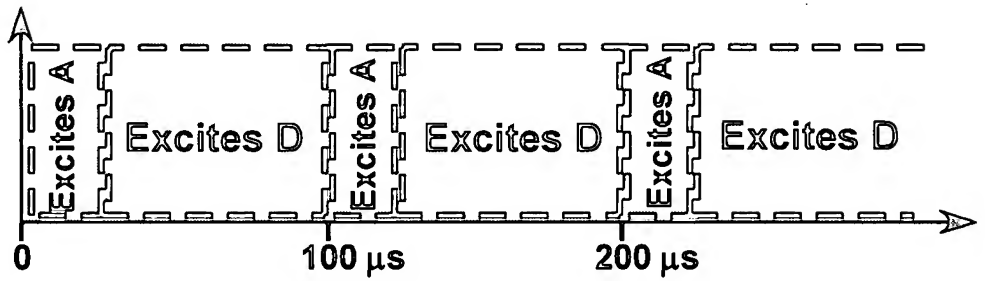


LIMITATIONS OF SINGLE-LASER EXCITATION spFRET

- Complex FRET Acceptor photophysics
  - "Dark" states→D-only peak
  - Photobleaching→ D-only peak
  - Intermittency ("Blinking")
- Complex FRET Donor photophysics
  - Intermittency
  - Transient QY changes
- Limited discrimination ability in the FRET coordinate
  - FRET range of 0-0.3 not easily accessible
- Variable fluorescence contamination
  - Adds variable counts to D-only peak

sp-FRET USING ALTERNATE LASER EXCITATION (ALEX)

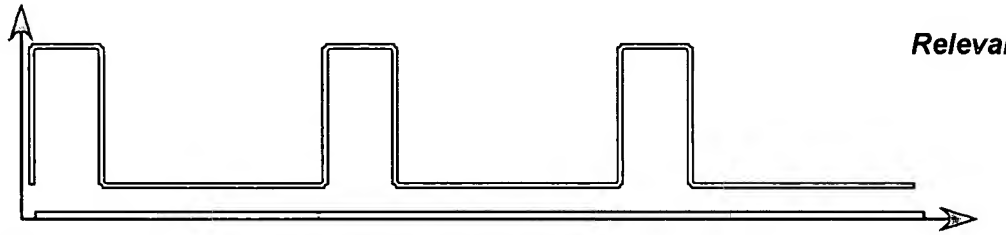
EXCITATION PROFILE



EMISSION PROFILES

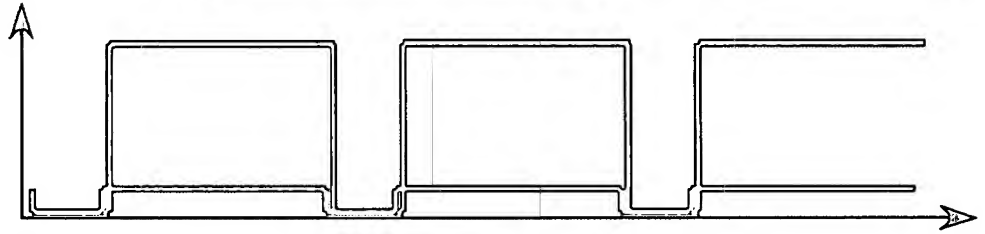
Relevant transcription species

A-only species



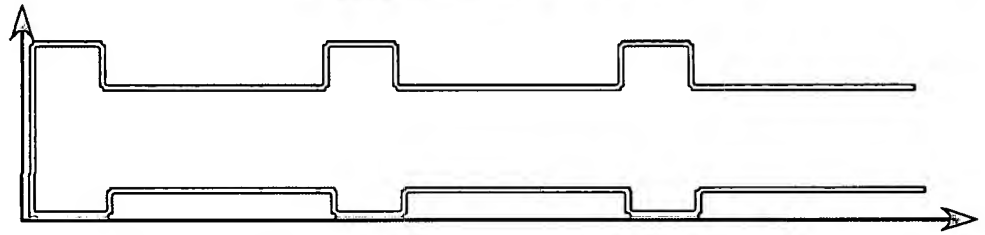
DNA^{A}

D-only species



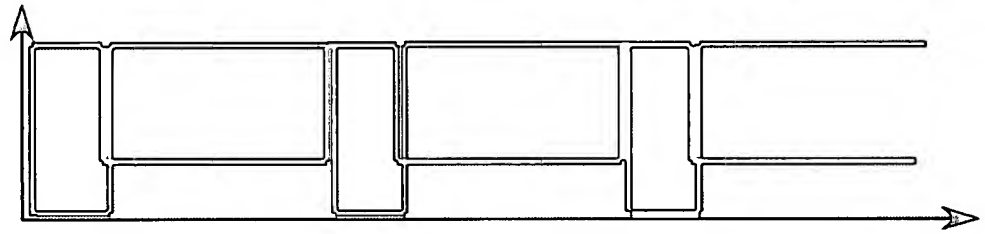
$RNAP\sigma^{D}$

D-A species, high FRET



RPo^{D-A}, Trailing-edge

D-A species low FRET



RPo^{D-A}, Leading-edge

EQUATIONS

Energy transfer ratio (*E*)

$$E = \frac{F^{DA}_{670em,514ex}}{F^{DA}_{670em,514ex} + F^{DA}_{580em,514ex}}$$

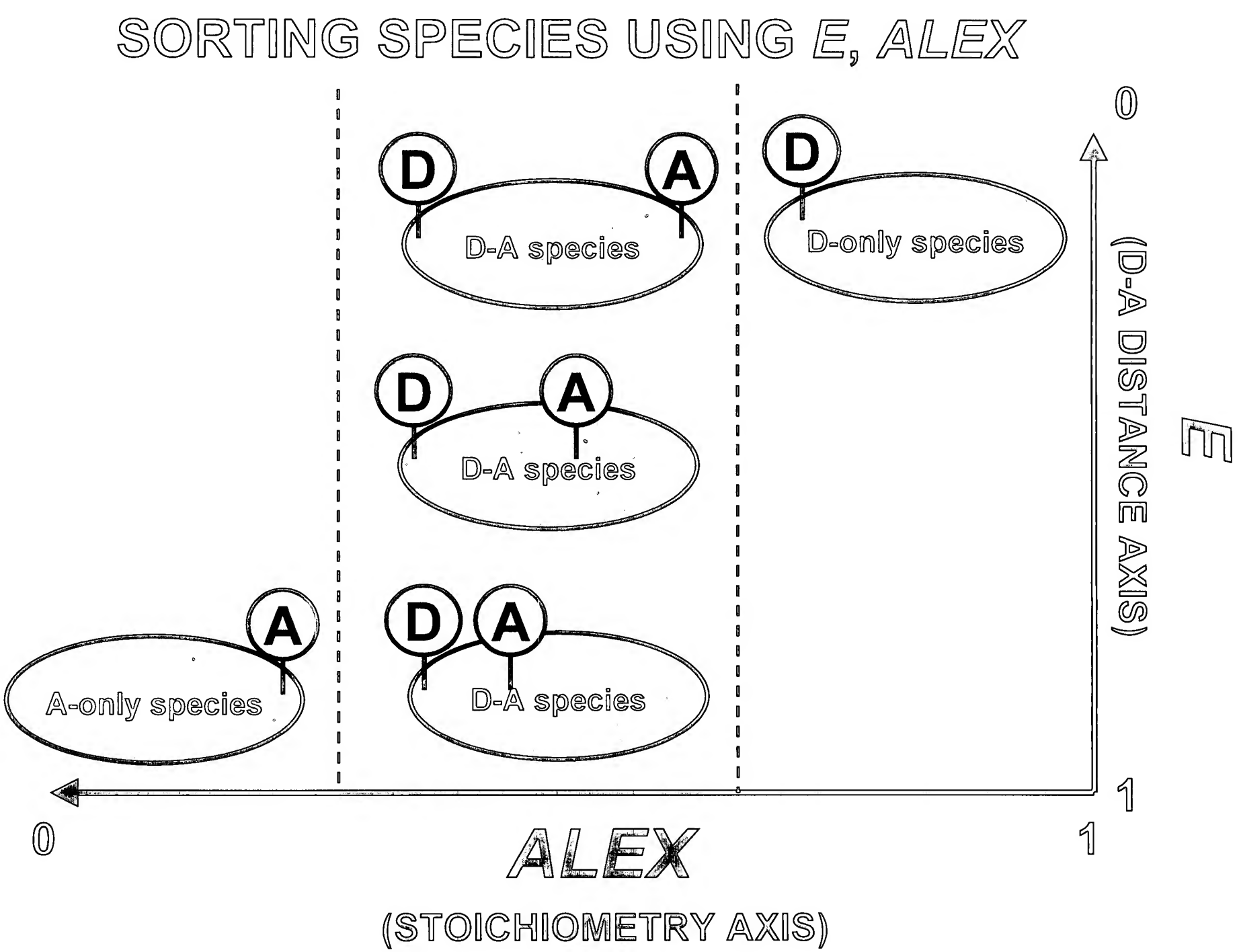
ALEX-based ratio (*ALEX*)

$$ALEX = \frac{F_{514ex}}{F_{514ex} + F_{638ex}} = \frac{F_{670em,514ex} + F_{580em,514ex}}{F_{670em,514ex} + F_{580em,514ex} + F_{670em,633ex}}$$

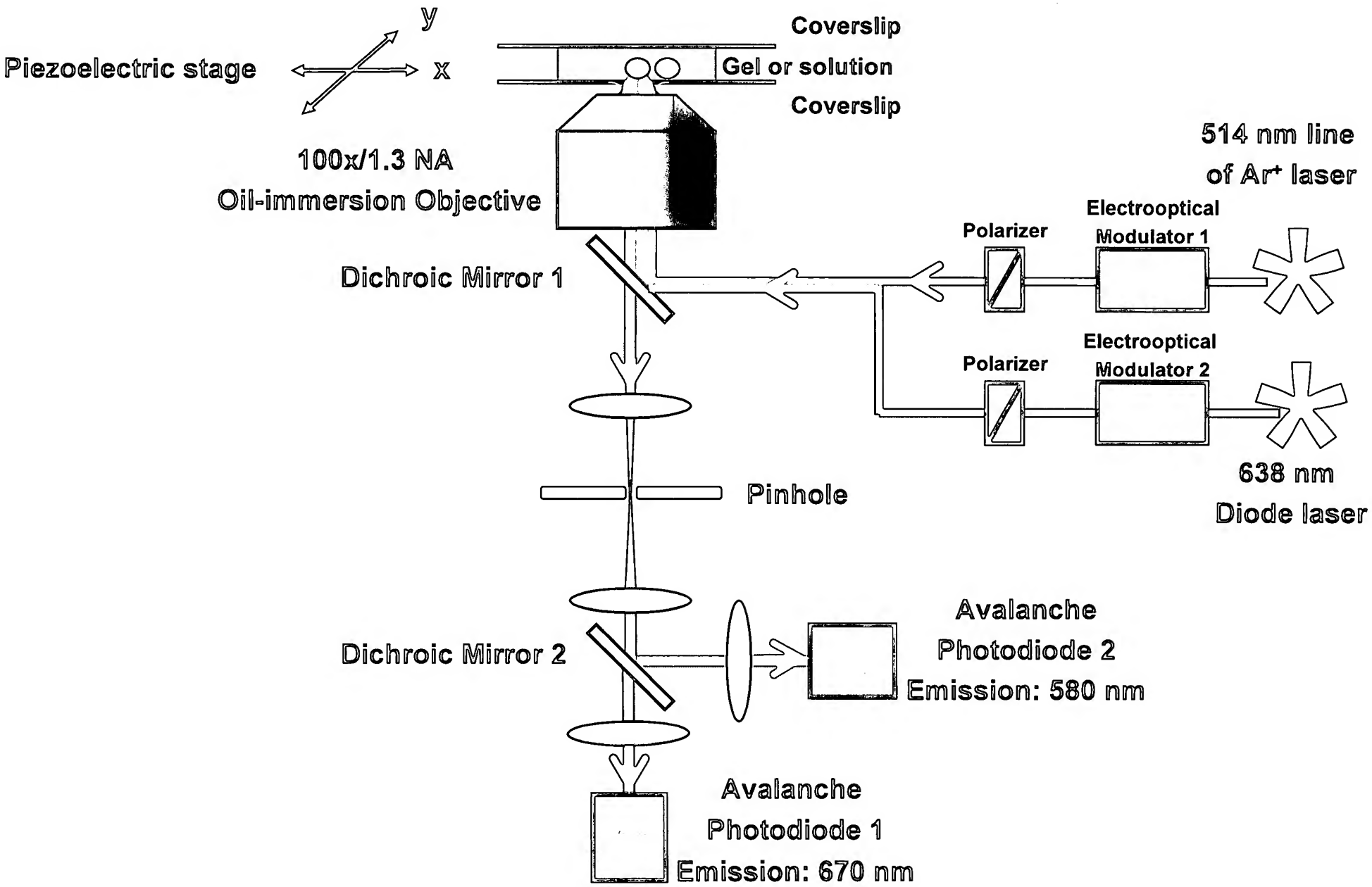
$$ALEX = \frac{0+100}{0+100+0} \sim 1.0$$

$$ALEX = \frac{50+50}{50+50+100} \sim 0.5$$

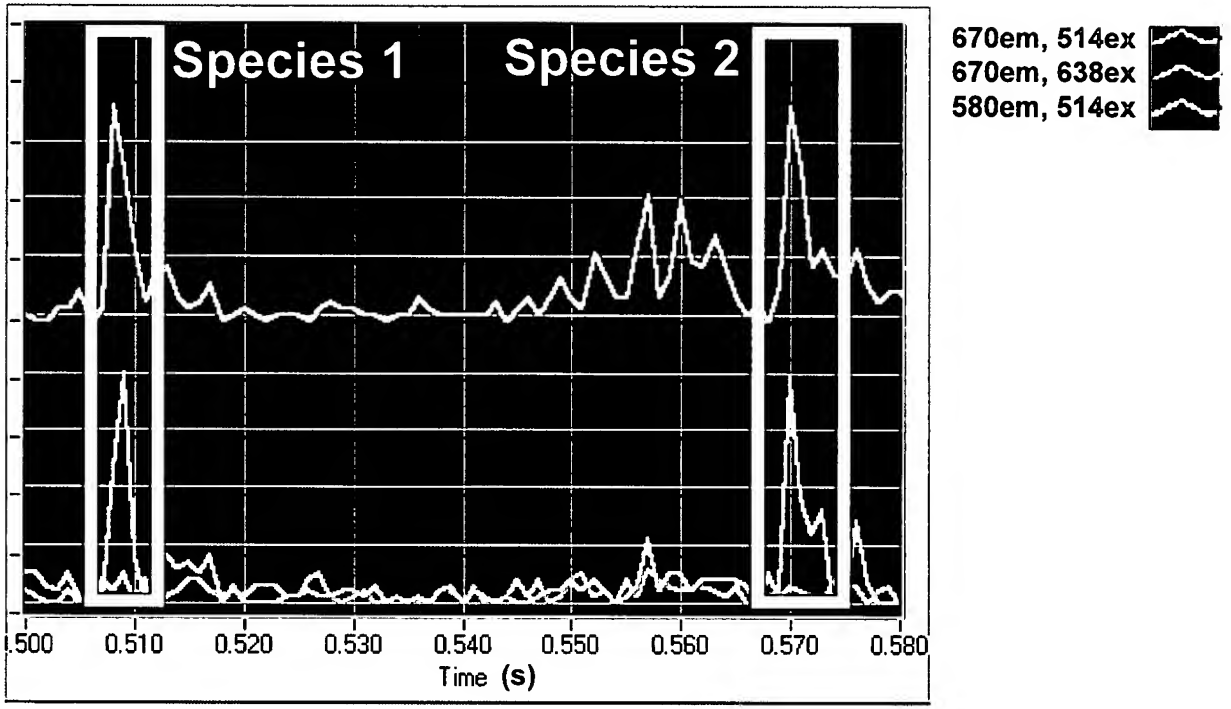
$$ALEX = \frac{0+0}{0+0+100} \sim 0.0$$


SORTING SPECIES USING E, ALEX
D
A
D-A species
D
D-only species
D
A
D-A species
A
A-only species
D
A
D-A species
0
(D-A DISTANCE AXIS)
E
1
0
ALEX
1
(STOICHIOMETRY AXIS)

ALEX SINGLE-MOLECULE CONFOCAL MICROSCOPY

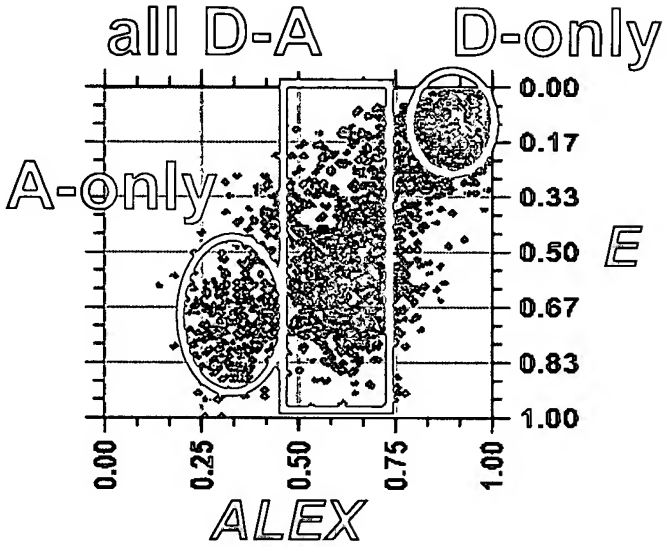


DATA ANALYSIS FOR INDIVIDUAL SPECIES

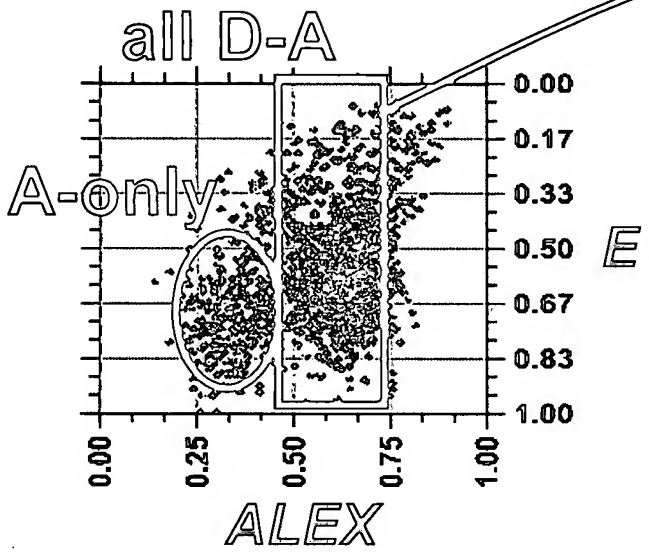


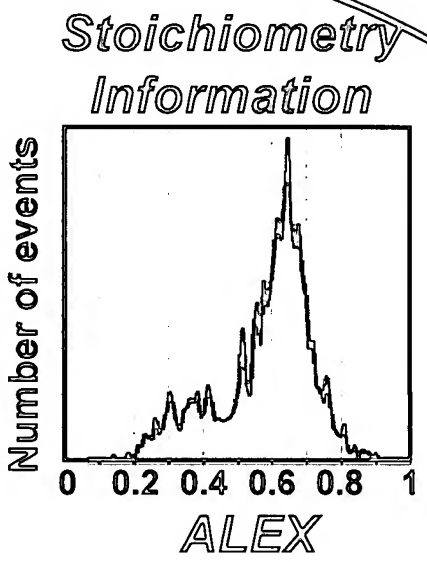
	Species 1	Species 2
670em, 514ex	71	85
670em, 638ex	69	93
580em, 514ex	7	11
FRET-sensitized A	52	60
E, simplified	91%	88%
E, FRET-sensitized A	91%	77%
ALEX	0.49	0.66

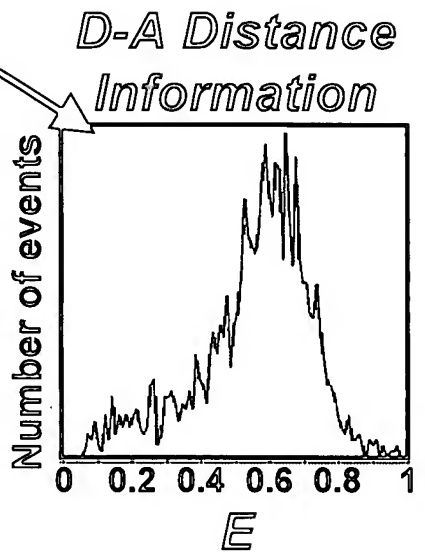
DATA ANALYSIS USING *E-ALEX* 2-D HISTOGRAMS



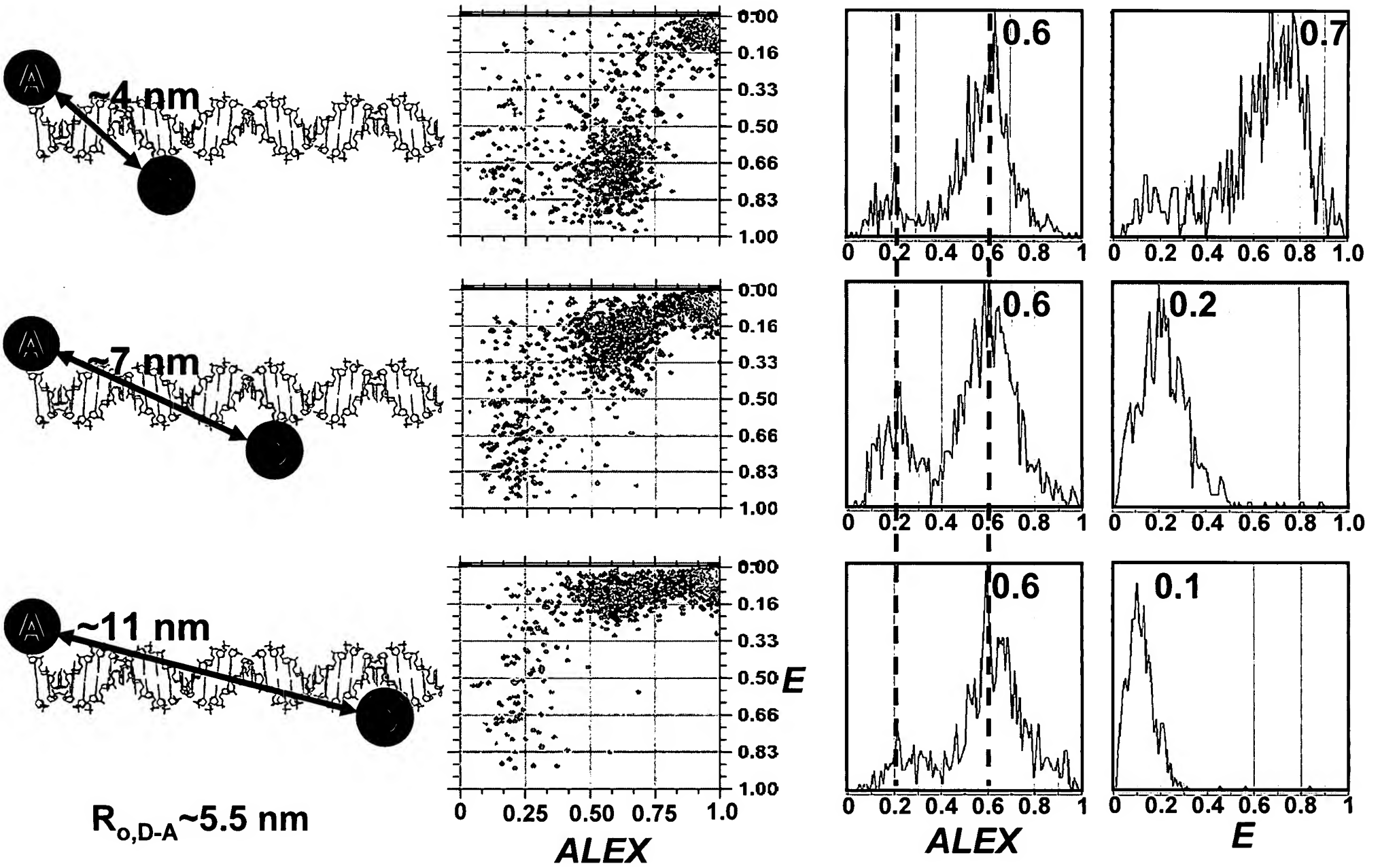
$F_{670em,638ex}$>15 KHz



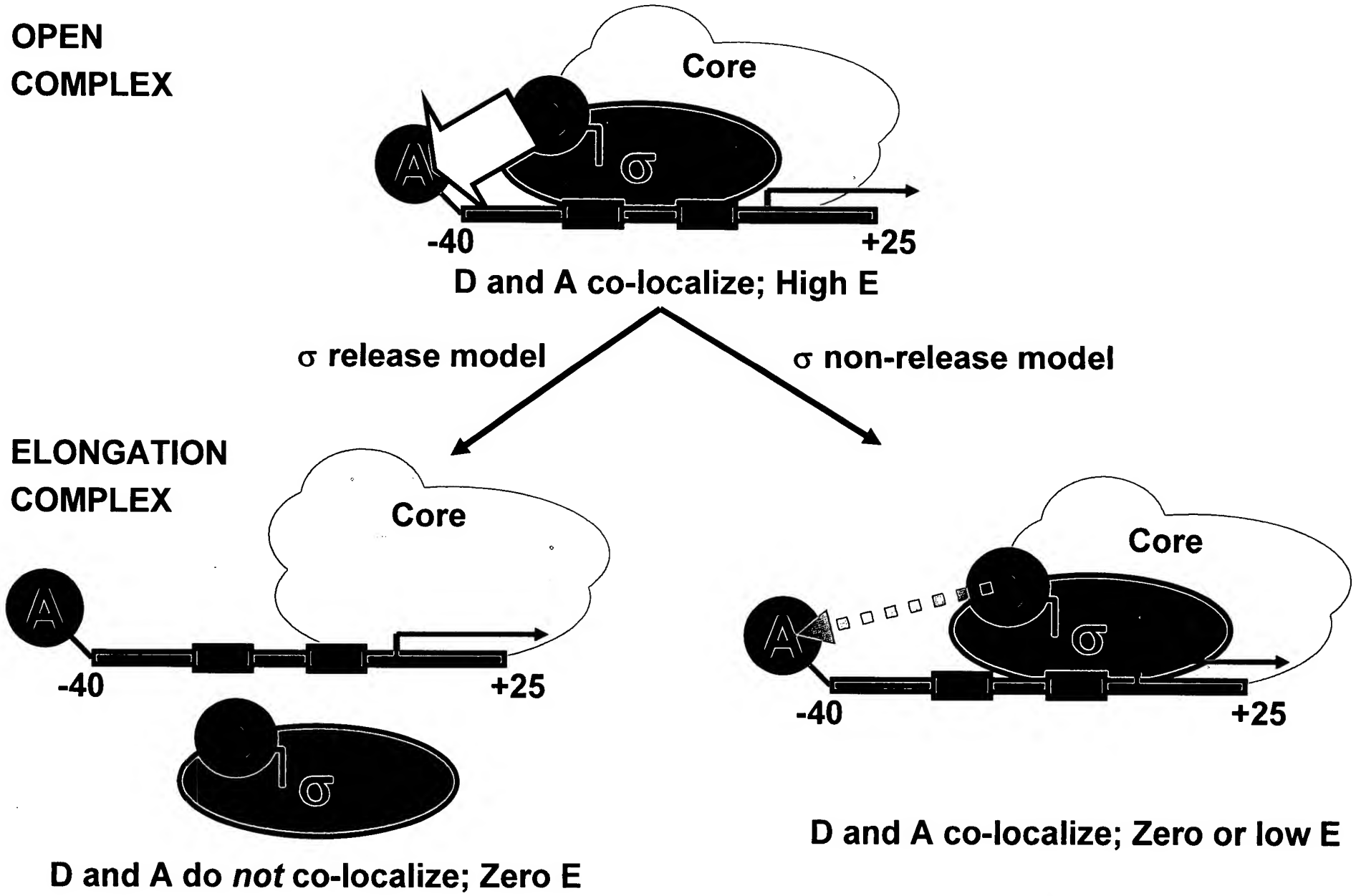




MODEL SYSTEMS: dsDNA



USING TRAILING-EDGE sp-FRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE



Mukhopadhyay *et al.*, 2001

TRAILING-EDGE spFRET

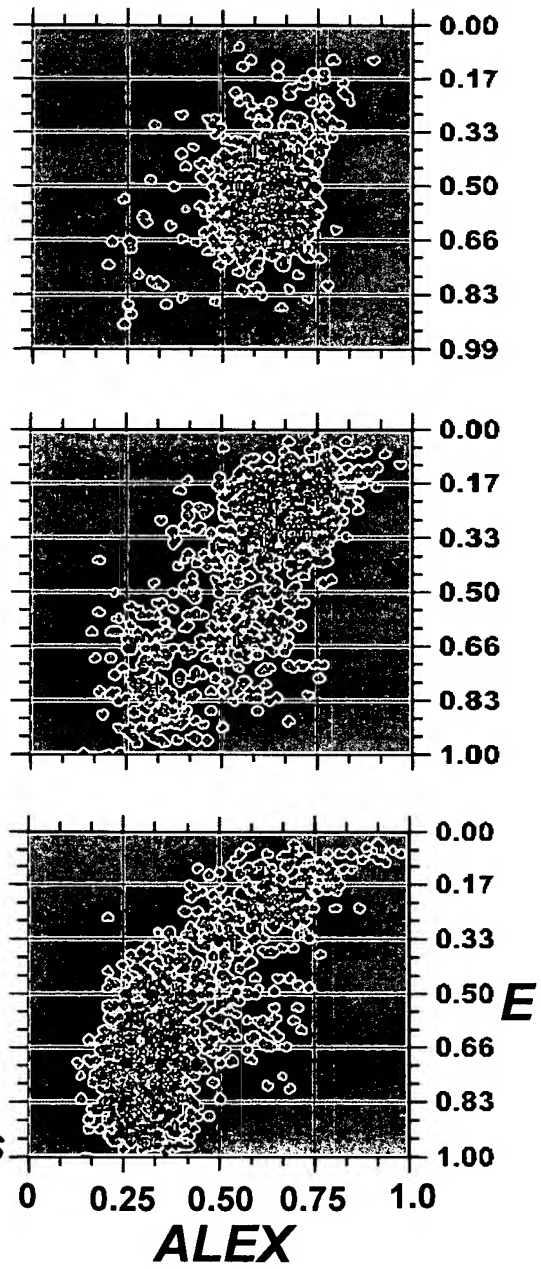
RNAP$\sigma^{TMR,569}$→lacUV5-11$^{Cy5,-40}$

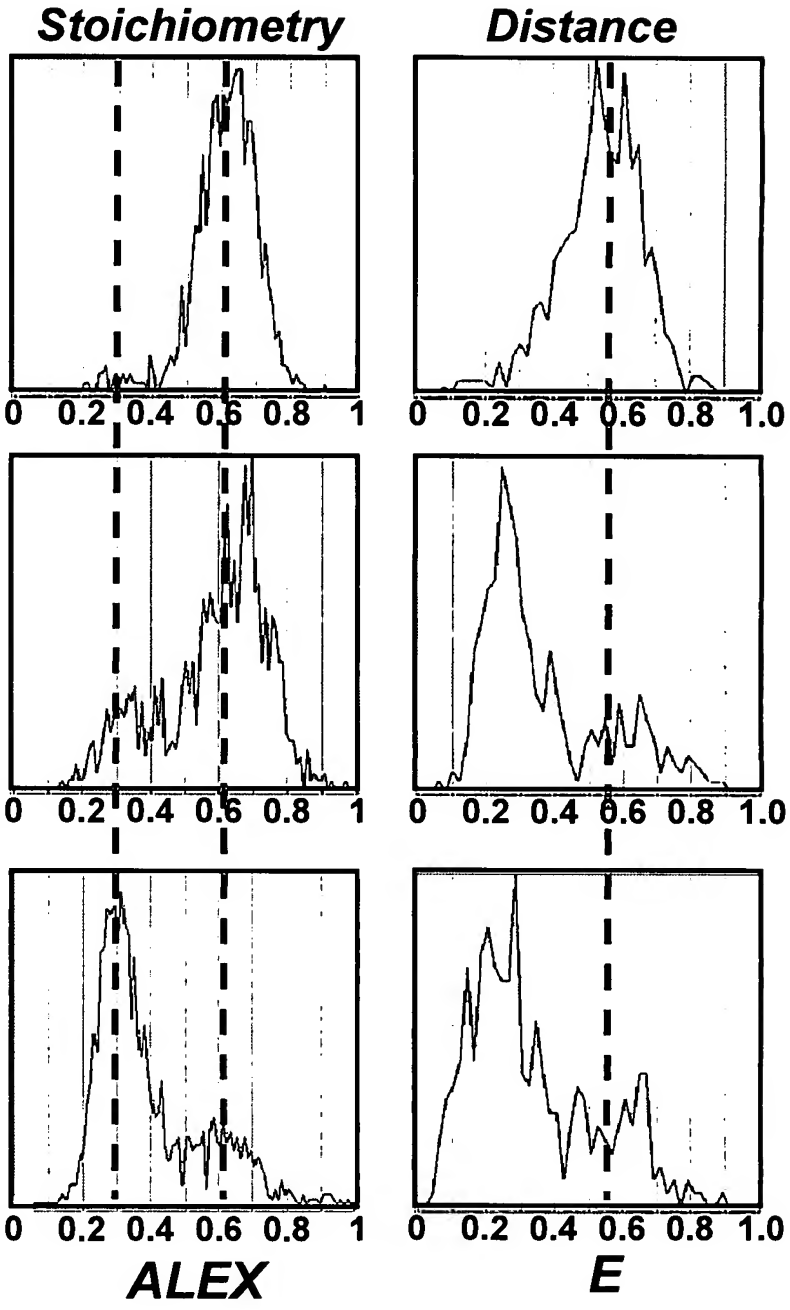
RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

RPo + ApA
+ 60 μM NTPs
(chase)

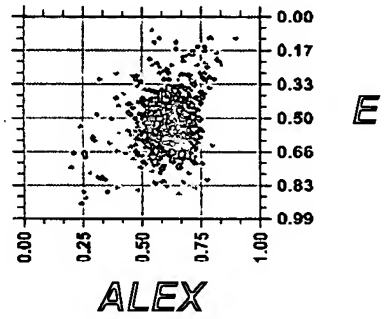
ABILITY OF STALLED COMPLEXES TO RESUME TRANSCRIPTION



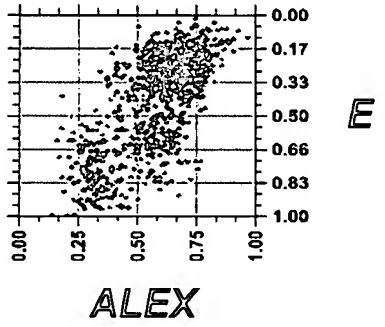


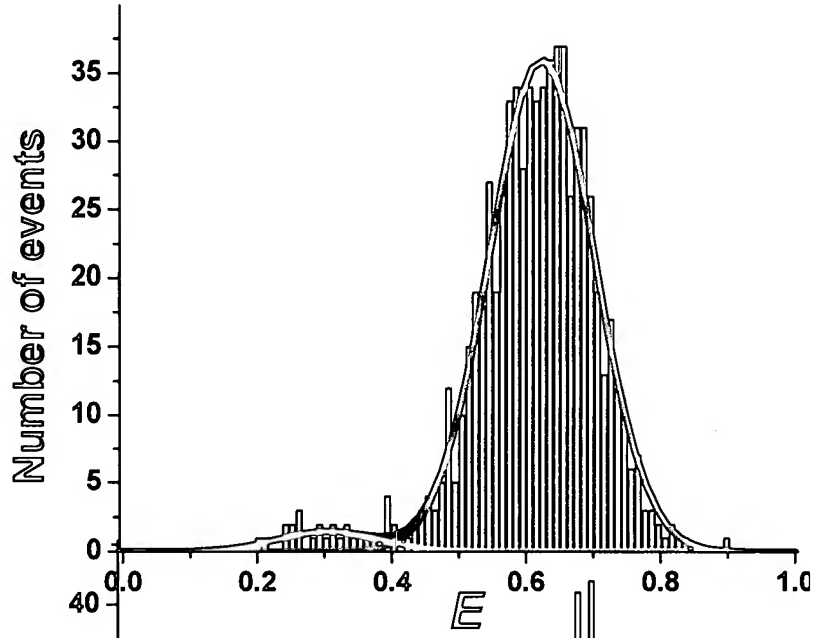
DIRECT OBSERVATION OF SIGMA NON-RELEASE: TRAILING-EDGE spFRET

$RP_{itc,2}$

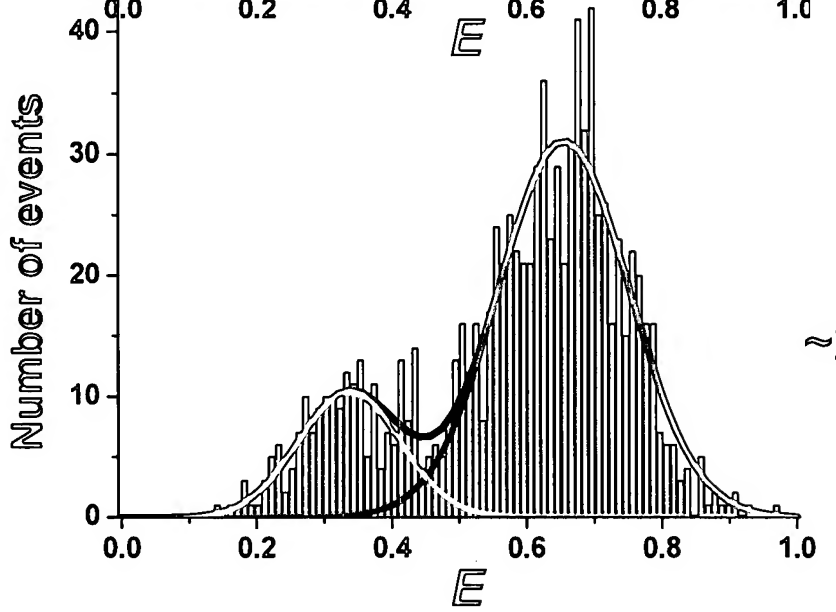


$RD_{e,11}$





~5% dissociation

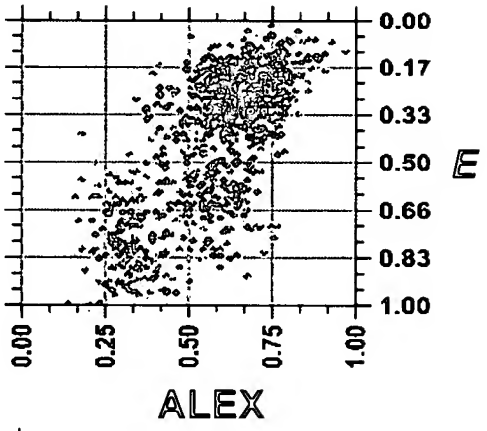


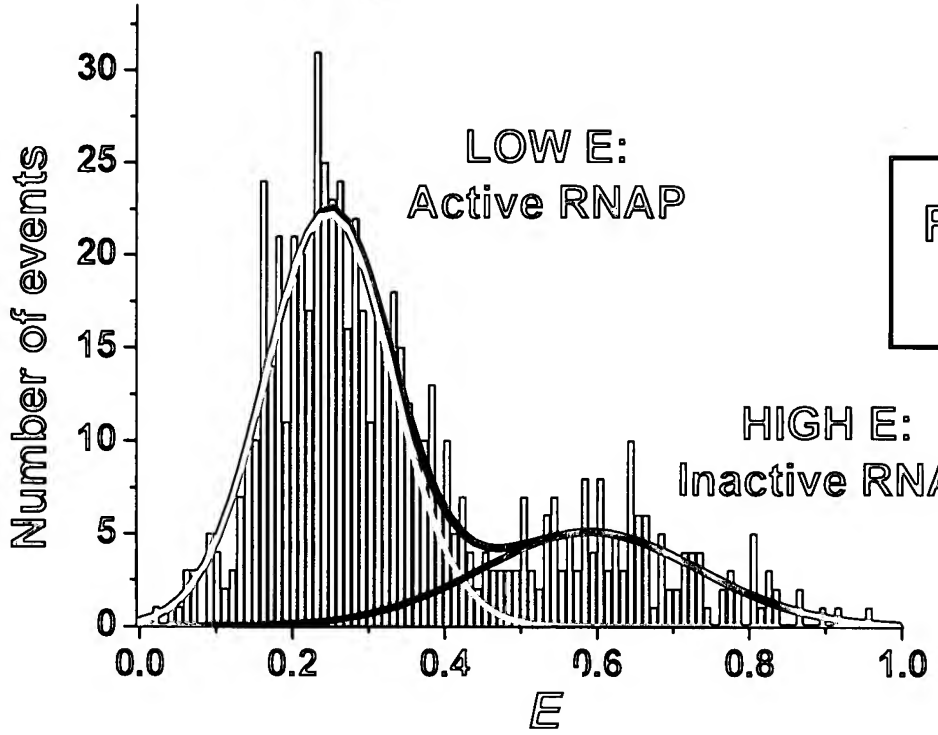
~20% dissociation

$$\% \text{ Dissociation} = \frac{(\text{A-only})}{(\text{A-only})+(\text{all D-A})}$$

E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: TRAILING-EDGE spFRET

RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

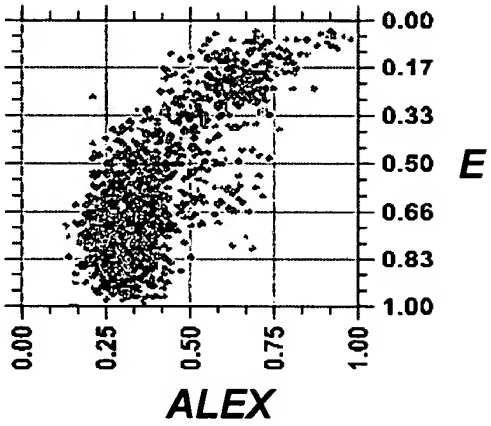


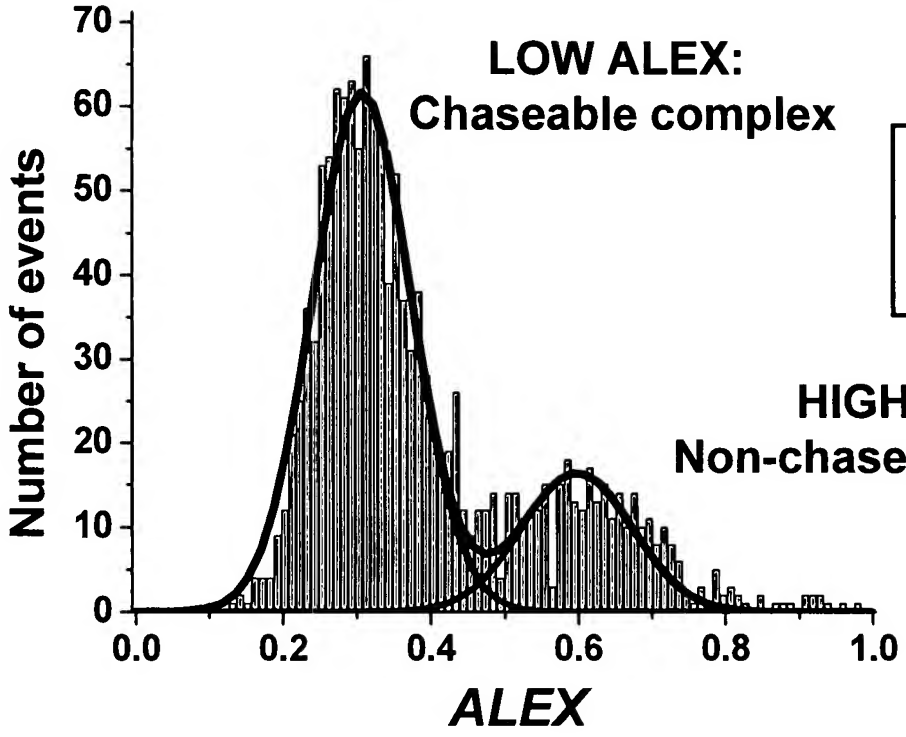


RNAP translocational activity ~ 70%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": TRAILING-EDGE spFRET

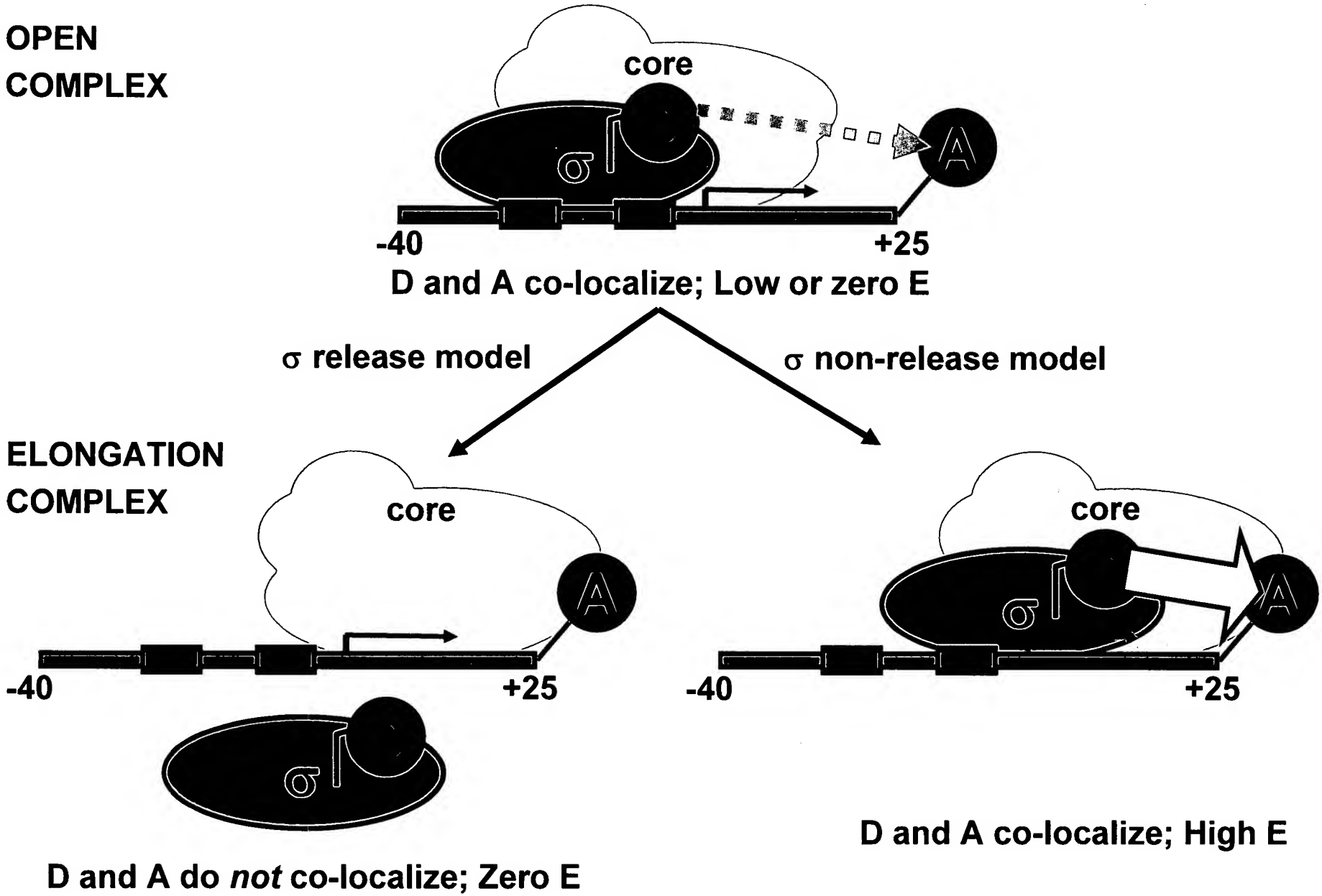
RPo + ApA + 60 μM NTPs (chase)





RNAP "chaseability" ~ 80%

USING LEADING-EDGE spFRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE



Mukhopadhyay *et al.*, 2001

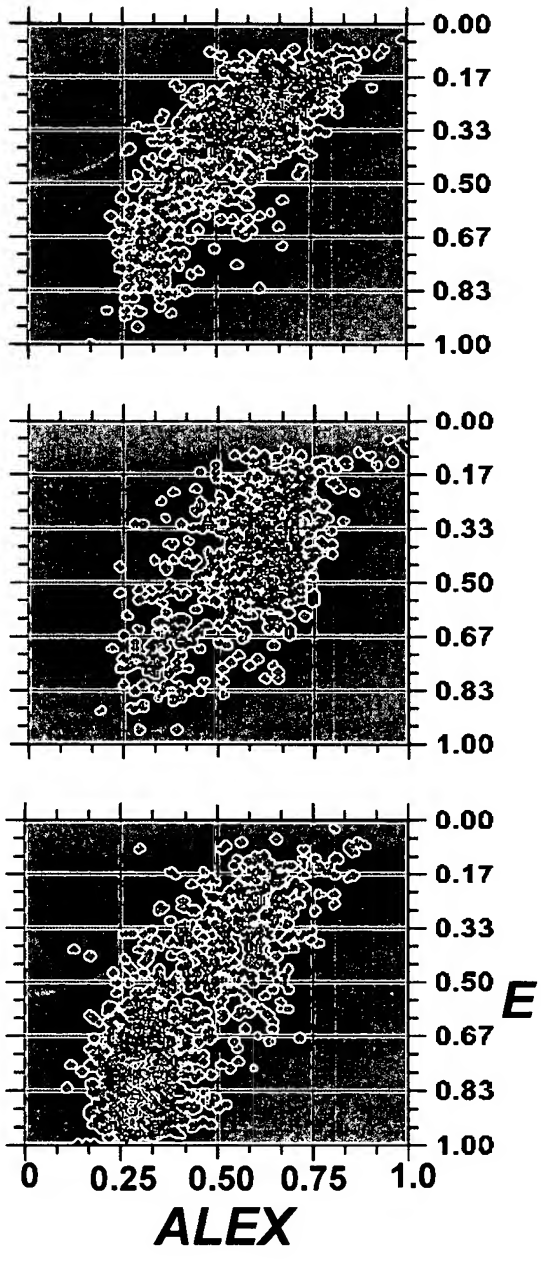
LEADING-EDGE spFRET

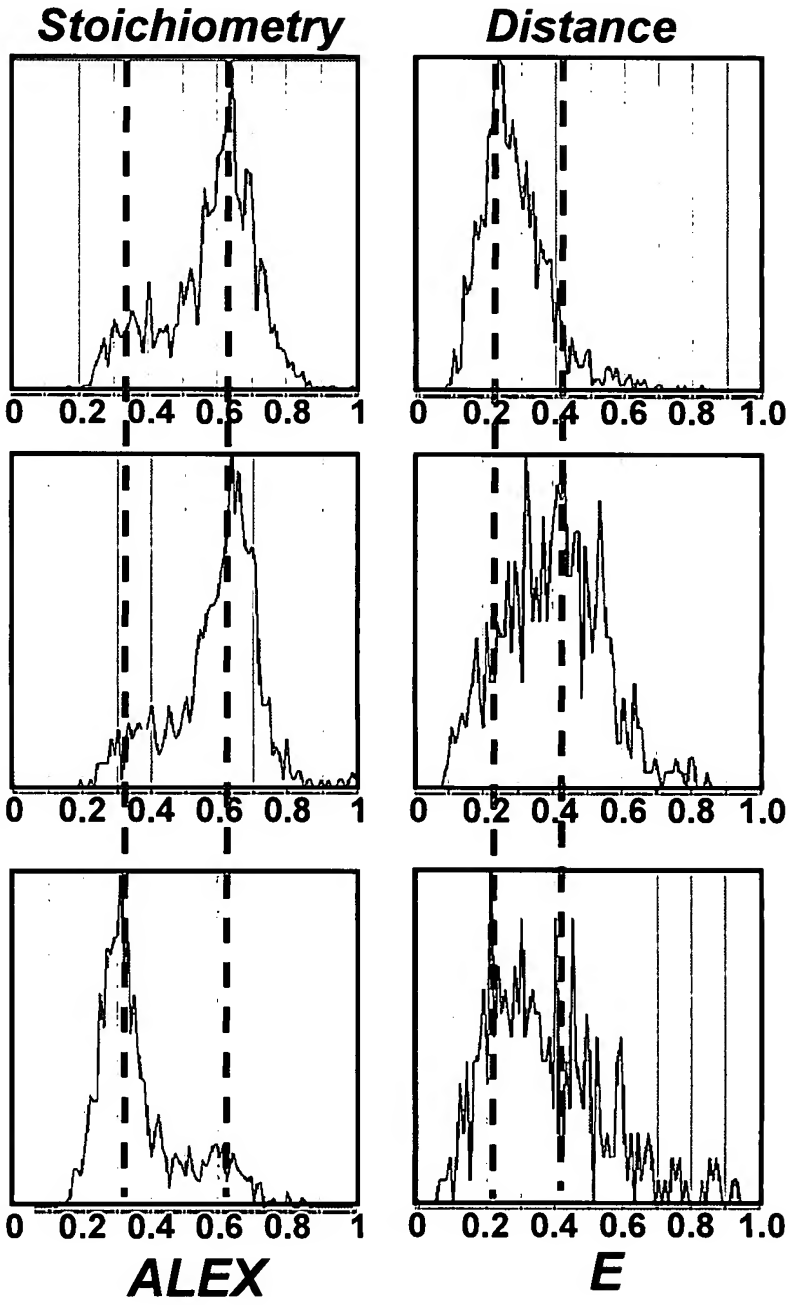
$RNAP\sigma^{TMR,366} \rightarrow lacUV5\text{-}11^{Cy5,+25}$

RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

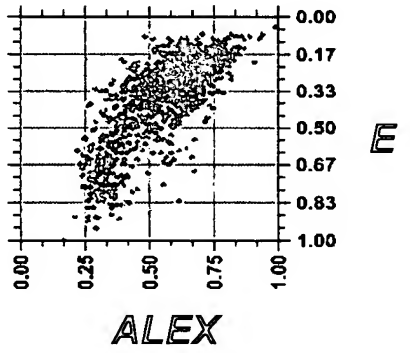
RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

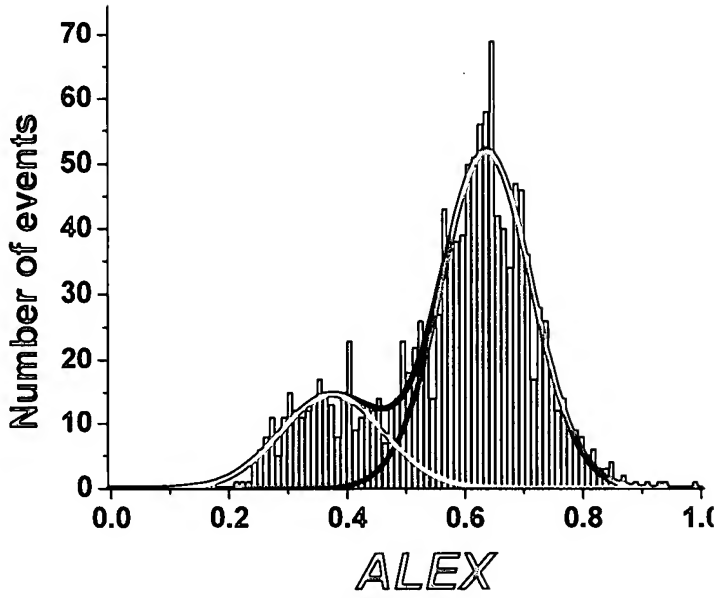
RPo + ApA
+ 60 μM NTPs
(chase)



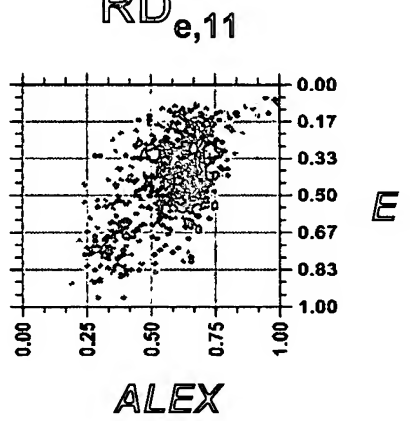


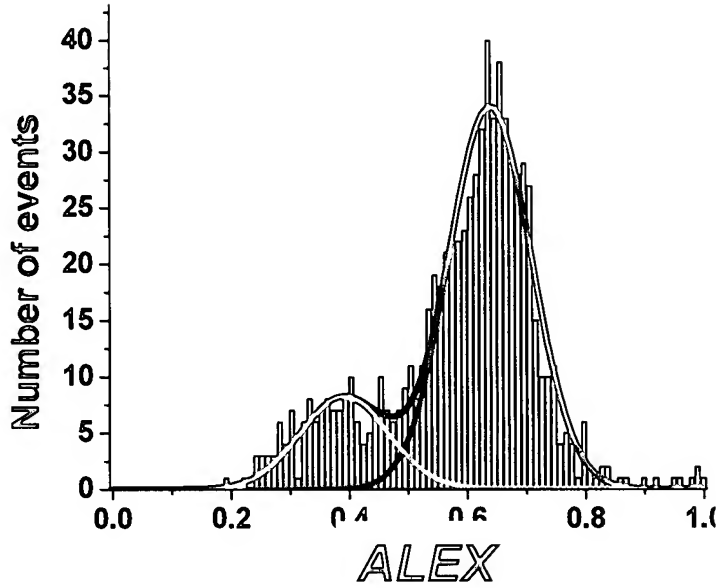
DIRECT OBSERVATION OF SIGMA NON-RELEASE: LEADING-EDGE spFRET





~20% dissociation

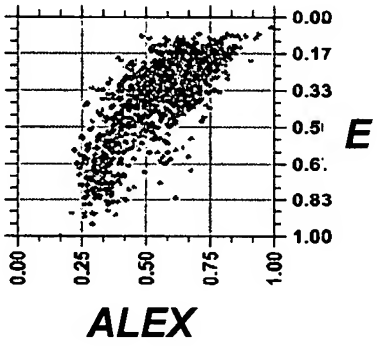


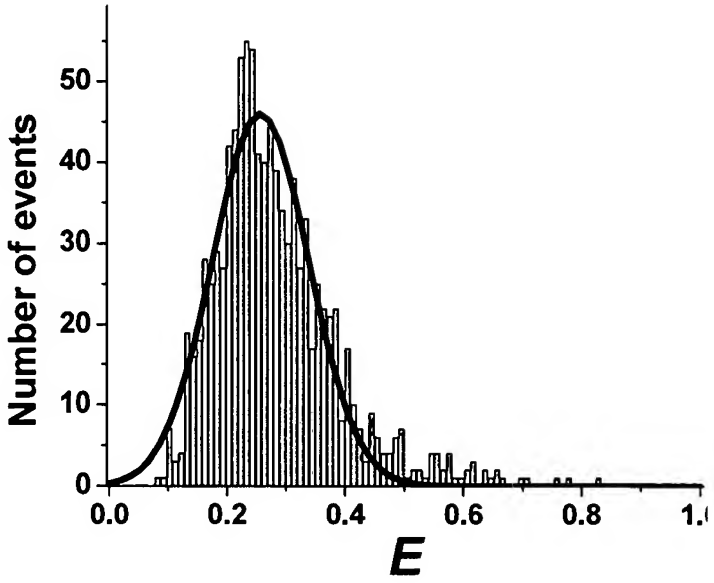


~20% dissociation

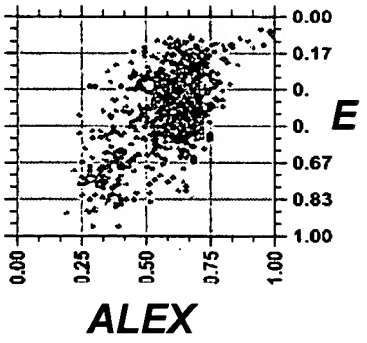
E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: LEADING-EDGE spFRET

RPo + ApA ($RP_{itc,2}$)



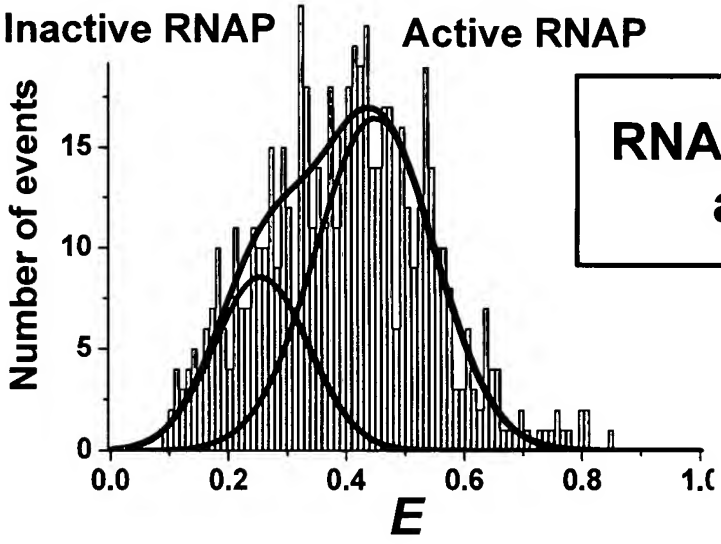


RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)



LOW E: Inactive RNAP

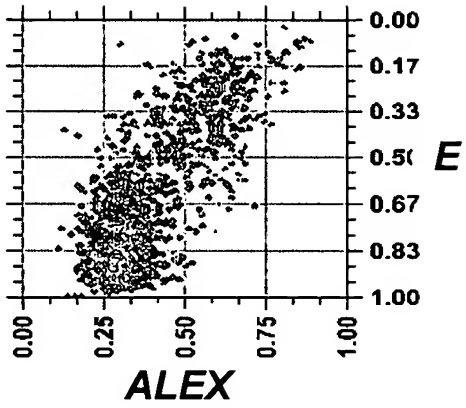
HIGH E: Active RNAP

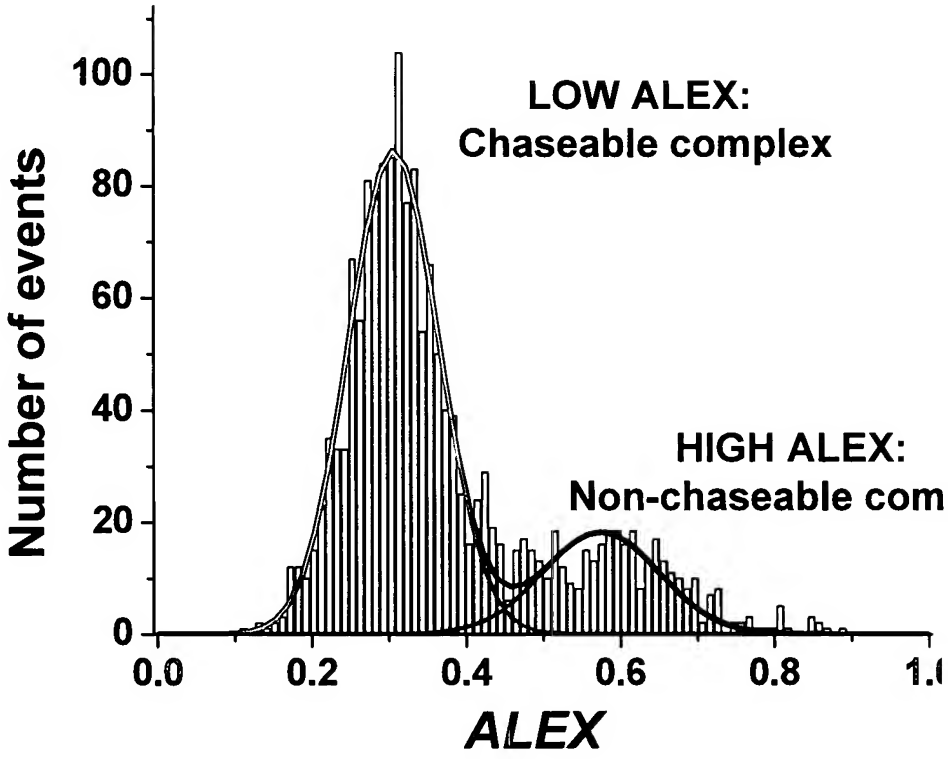


RNAP translocational activity = 72%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": LEADING-EDGE spFRET

RPo + ApA + 60 μM NTPs (chase)



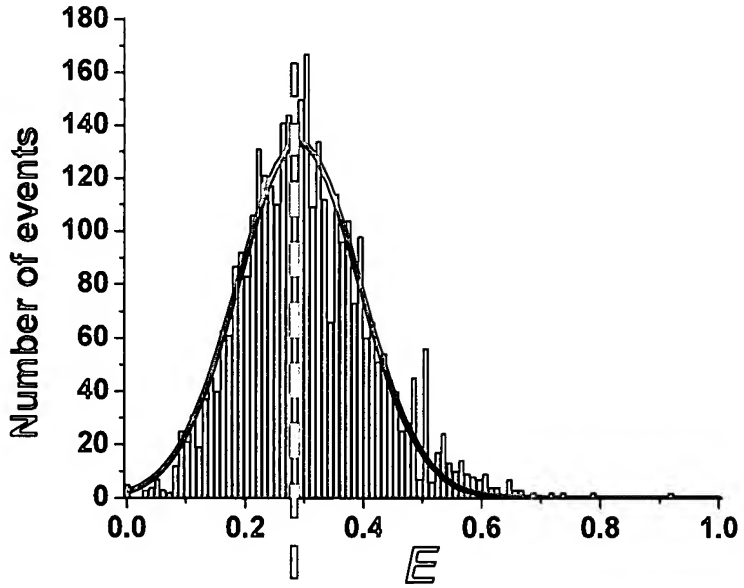


RNAP "chaseability" = 80%

LEADING-EDGE spFRET DETECTS MOVEMENT OF LEADING EDGE DURING ABORTIVE INITIATION

RPo + ApA

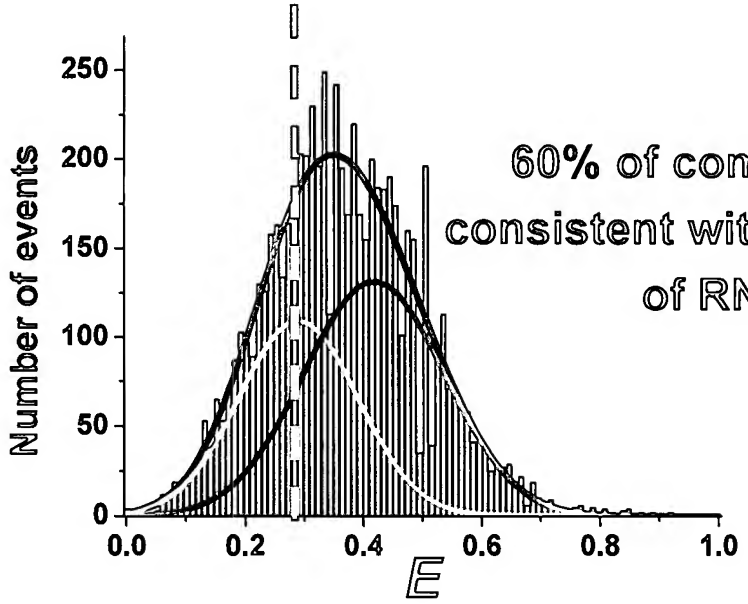
($RP_{itc,2}$)



RPo + ApA

+ 25 μM UTP/GTP

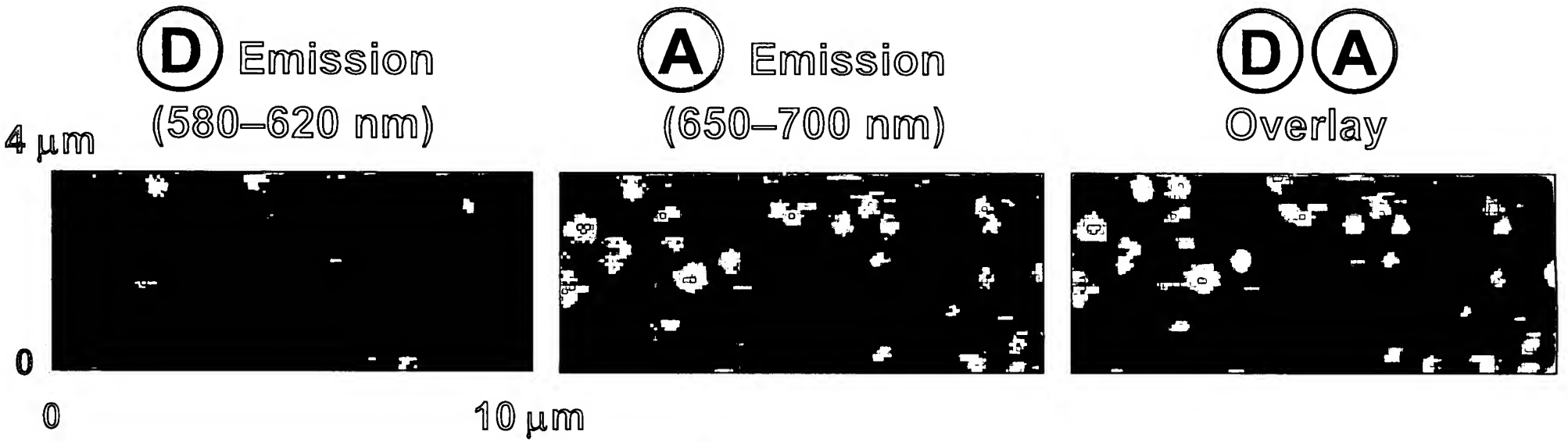
($RD_{e,7}$)



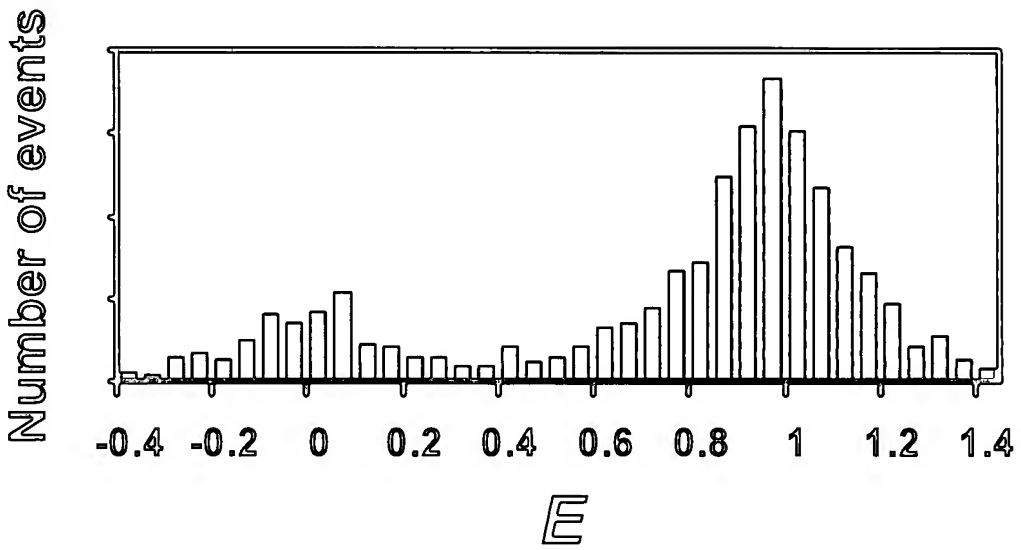
60% of complexes show higher *E*; consistent with downstream movement of RNAP leading edge

TRAILING-EDGE spFRET ON SURFACE-IMMOBILIZED RP_o COMPLEXES

Excitation: 514 nm line of Ar^+ laser

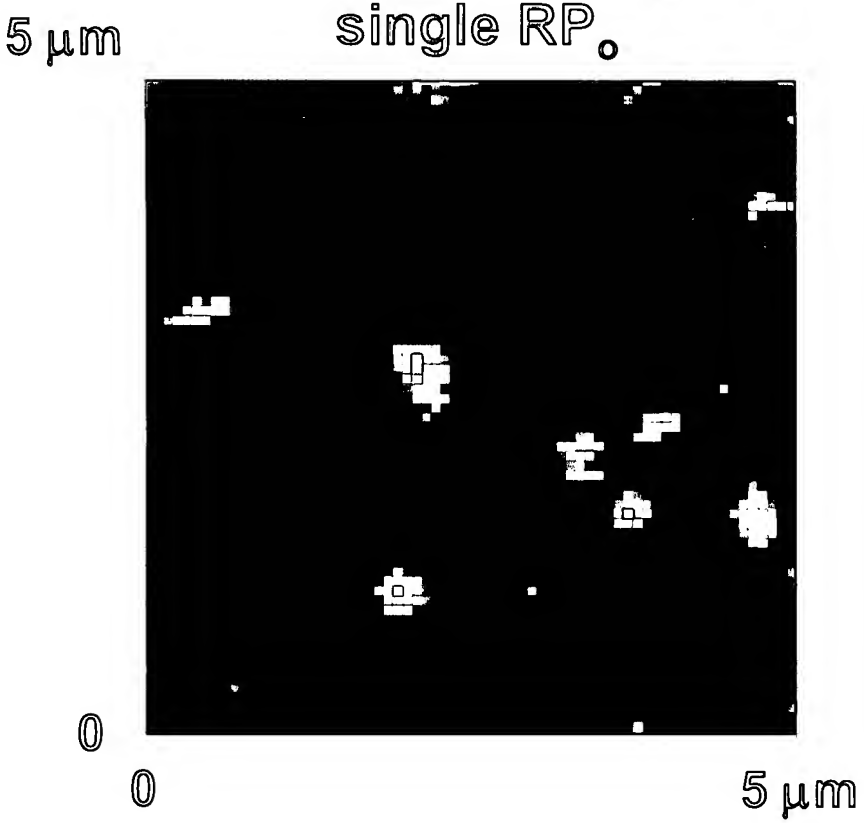


$$E = \frac{I_A}{I_A + \gamma I_D}$$

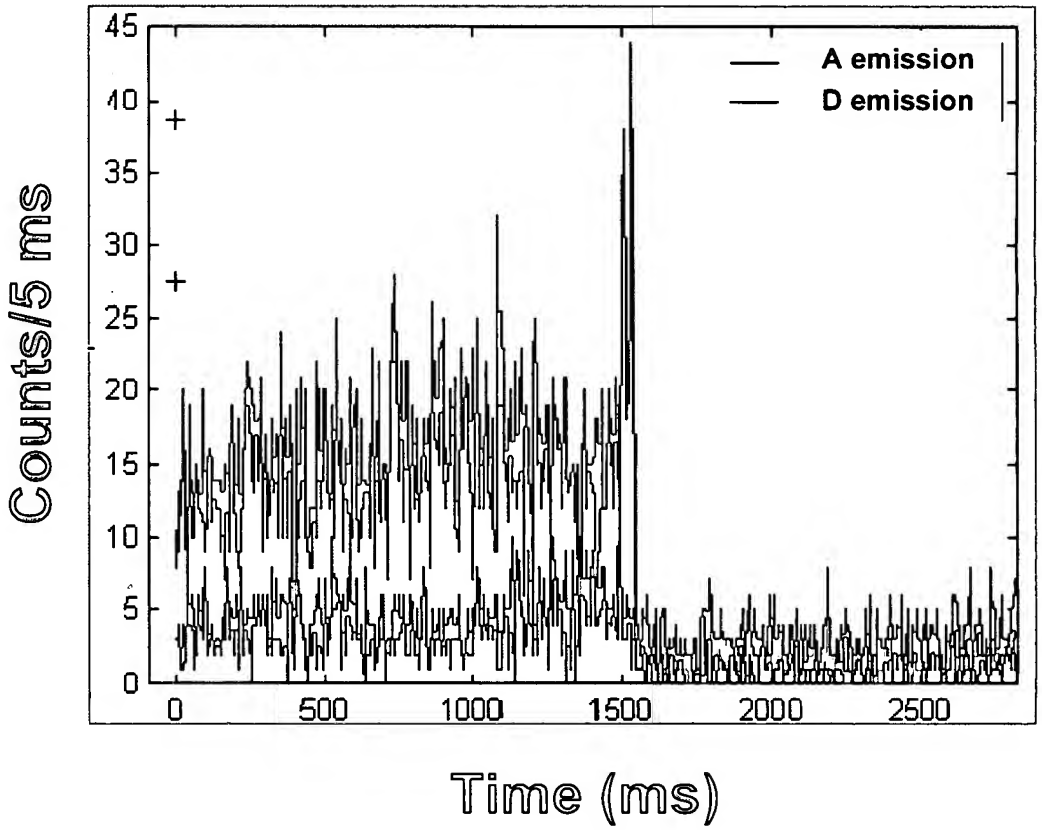


IMAGING AND TIME-TRAJECTORIES OF SINGLE RP_o COMPLEXES

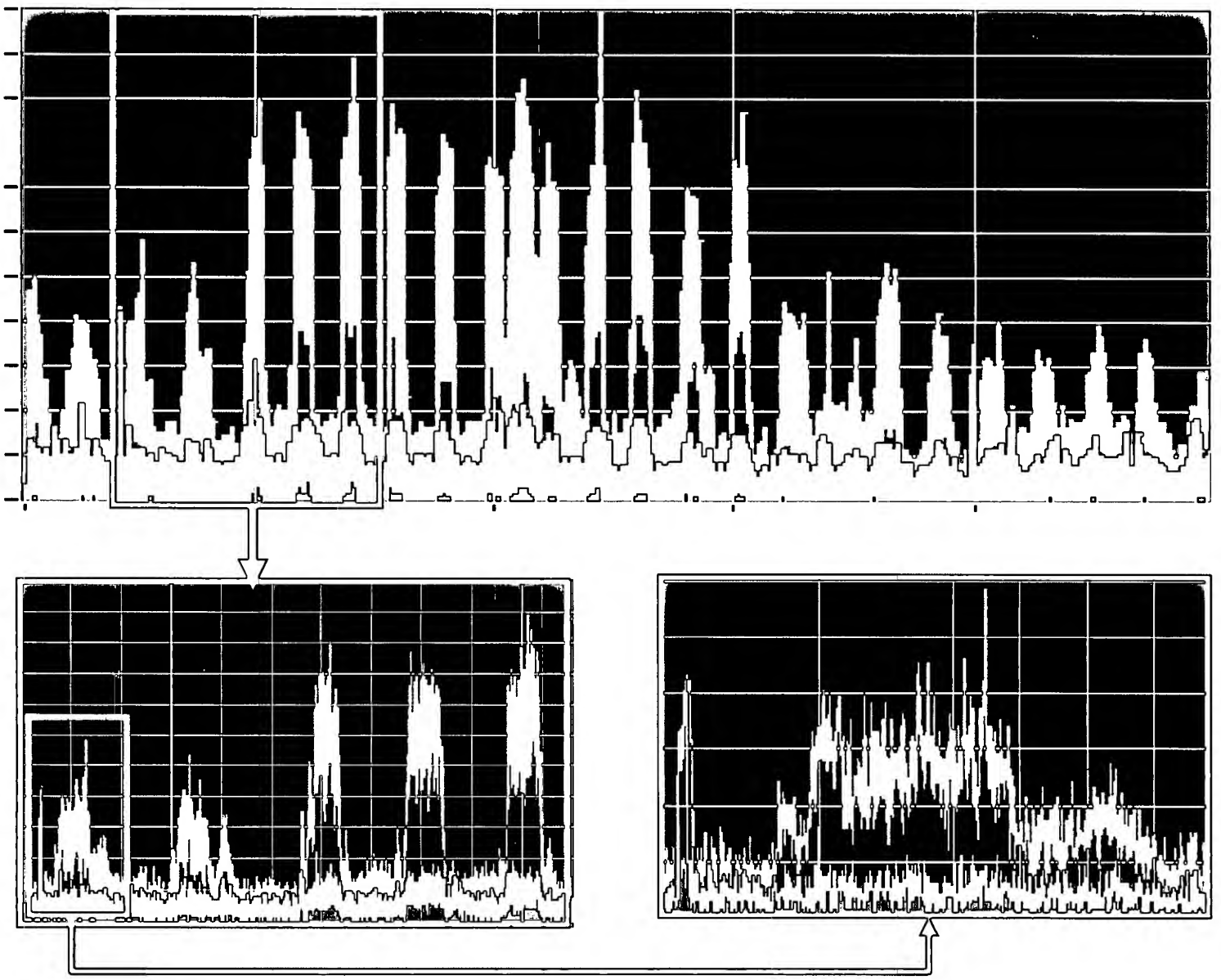
Single-step photobleaching: evidence for imaging single RP_o



Time-trajectory for a single RP_o showing TE-FRET



MONITORING SINGLE-ENZYME DYNAMICS ON IMMOBILIZED MOLECULES



CONCLUSIONS

- Developed robust assays for analysis of structure, dynamics, and activity of protein-DNA complexes
- Confirmed sigma presence in early elongation complexes
- Determined activity for translocation and for chase reactions
- Detected movement of leading edge during abortive initiation
- Future work:
    - Abortive initiation mechanism
    - Sigma dynamics at various transcription steps

ACKNOWLEDGEMENTS

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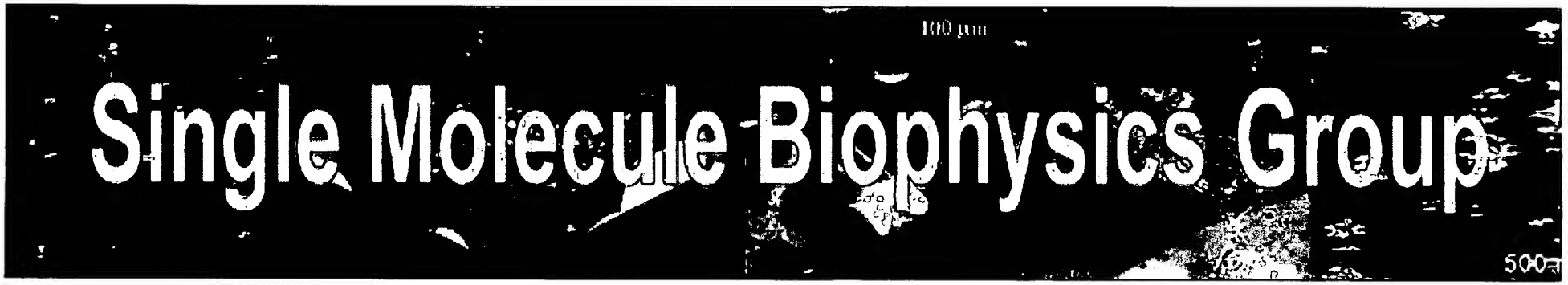
Ekaterine Kortkhonjia
Vladimir Mekler
Jayanta Mukhopadhyay
Andrey Revyakin

Philip Tinnefeld (U.Heidelberg)

and all SMBs!

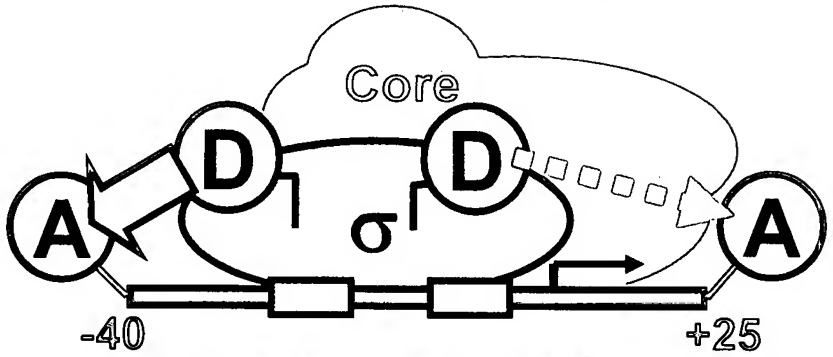


Funding: DOE, NIH

TRAILING-EDGE and LEADING-EDGE FRET:

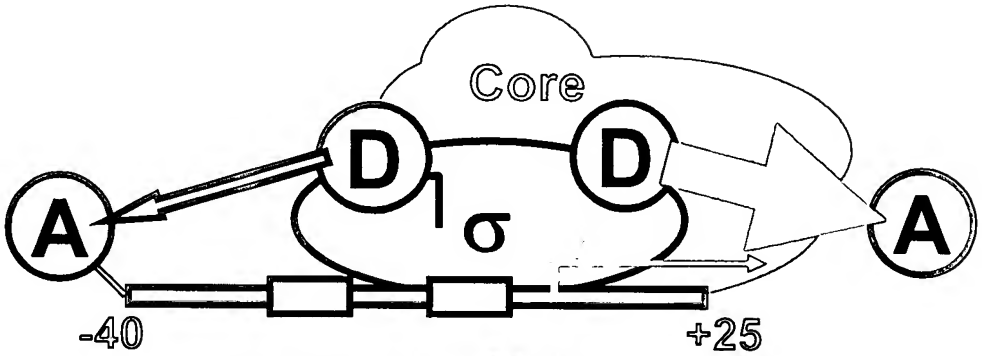
Assay of translocation of a protein relative to a nucleic acid

Trailing-edge/leading-edge FRET (TELE-FRET)



Ruler 1: High FRET

Ruler 2: Low FRET

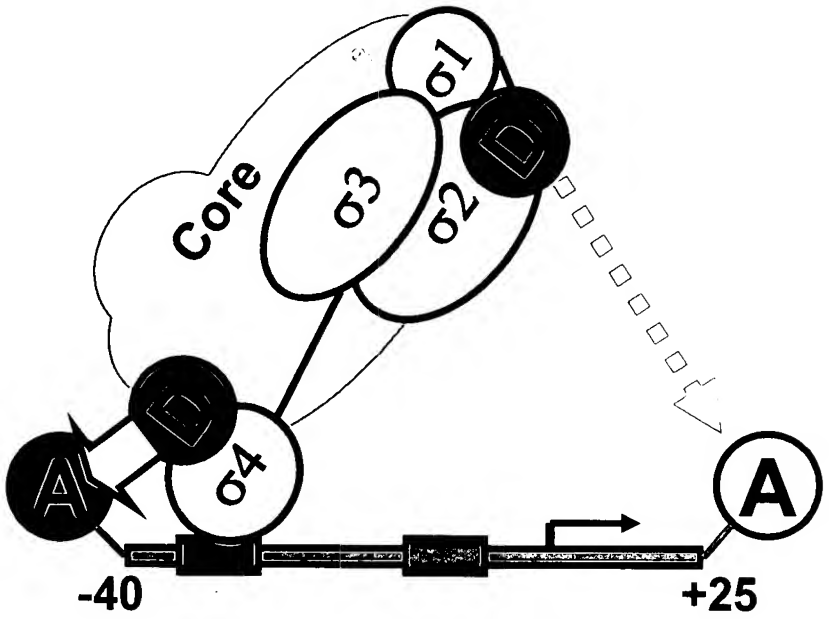


Ruler 1: Low FRET

Ruler 2: High FRET

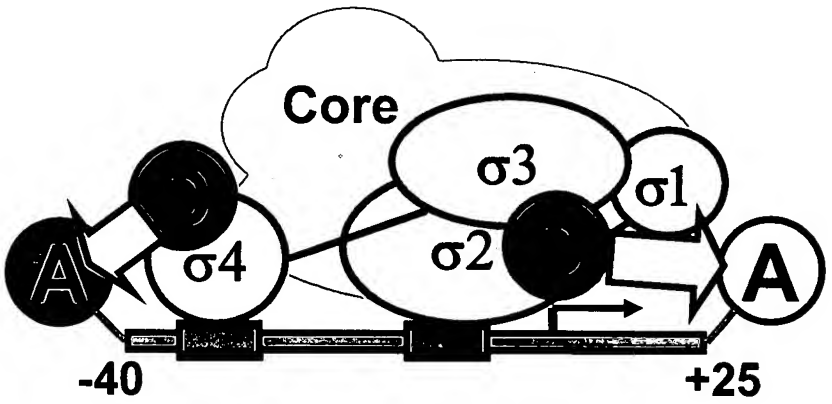
Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

Step-Sequence of formation of promoter contacts using 2 FRET rulers



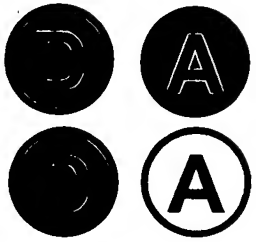
Ruler 1: High FRET

Ruler 2: Low FRET



Ruler 1: High FRET

Ruler 2: High FRET



Ruler 1

Ruler 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Shimon Weiss	Art Unit: 2877
Appl. No.: 10/561,448	Examiner: F.L. Evans
Confirmation No.: 8178	
Filed: December 20, 2005	Atty. Docket No.: 58086-226455
For: MODULATED EXCITATION FLUORESCENCE ANALYSIS	Customer No. 26694
	PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 C.F.R. § 1.131

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, being duly warned, declare the following:

1. I am a co-inventor of the subject matter described and claimed in the above-identified U.S. patent application. I have reviewed the claims of this application as currently amended.

2. I understand that the Office Action dated November 30, 2007 rejected the examined claims of this patent application under 35 U.S.C. § 102(a) over published German patent application Publication No. DE 10210737 A1 by Krieger et al. that published March 20, 2003.

3. I, together with my co-inventors, conceived the invention described and claimed in at least independent claims 1 and 21 of this application, and reduced it to practice, prior to the March 20, 2003 publication date of the cited reference. Our prior invention is evidenced by a copy of a presentation by one of the co-inventors, Achillefs Kapanidis, at the Single-Molecule Biophysics Conference in Aspen, CO on January 7, 2003, (copy attached as Exhibit A).

4. As documented by Exhibit A, my co-inventors and I conceived the invention of at least current independent claims 1 and 21, and reduced it to practice, prior to January 7, 2003.

5. The acts described above in paragraphs 3 and 4 were carried out in the United States of America, or else in a WTO member country.

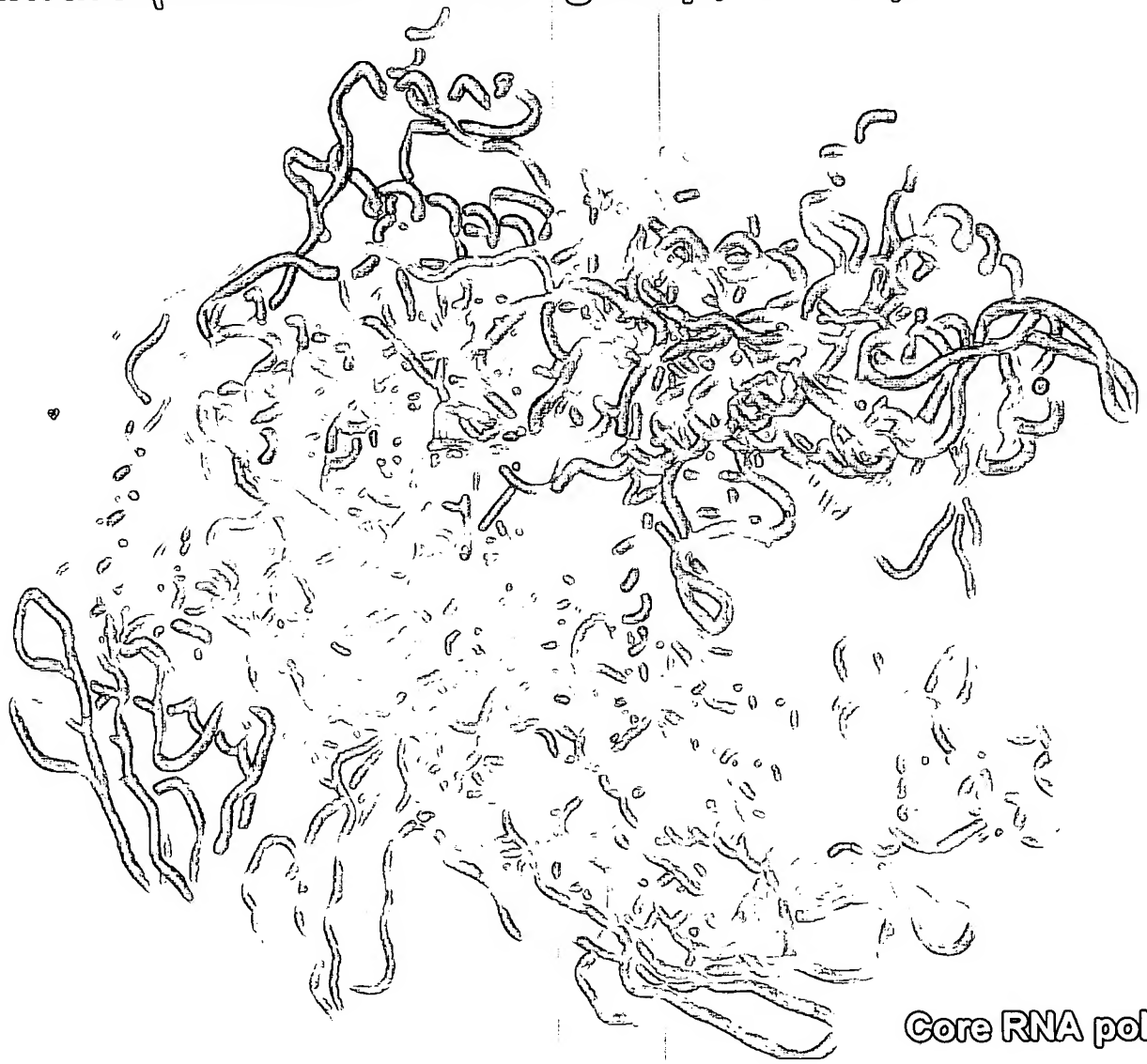
6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date	Shimon Weiss
Date	Achillefs Kapanidis
5/28/2008 Date	Ted A. Laurence Ted A. Laurence
Date	Nam K. Lee

Exhibit A

Molecular Machines at Work:
Single-Molecule Analysis of Transcription by RNA Polymerase

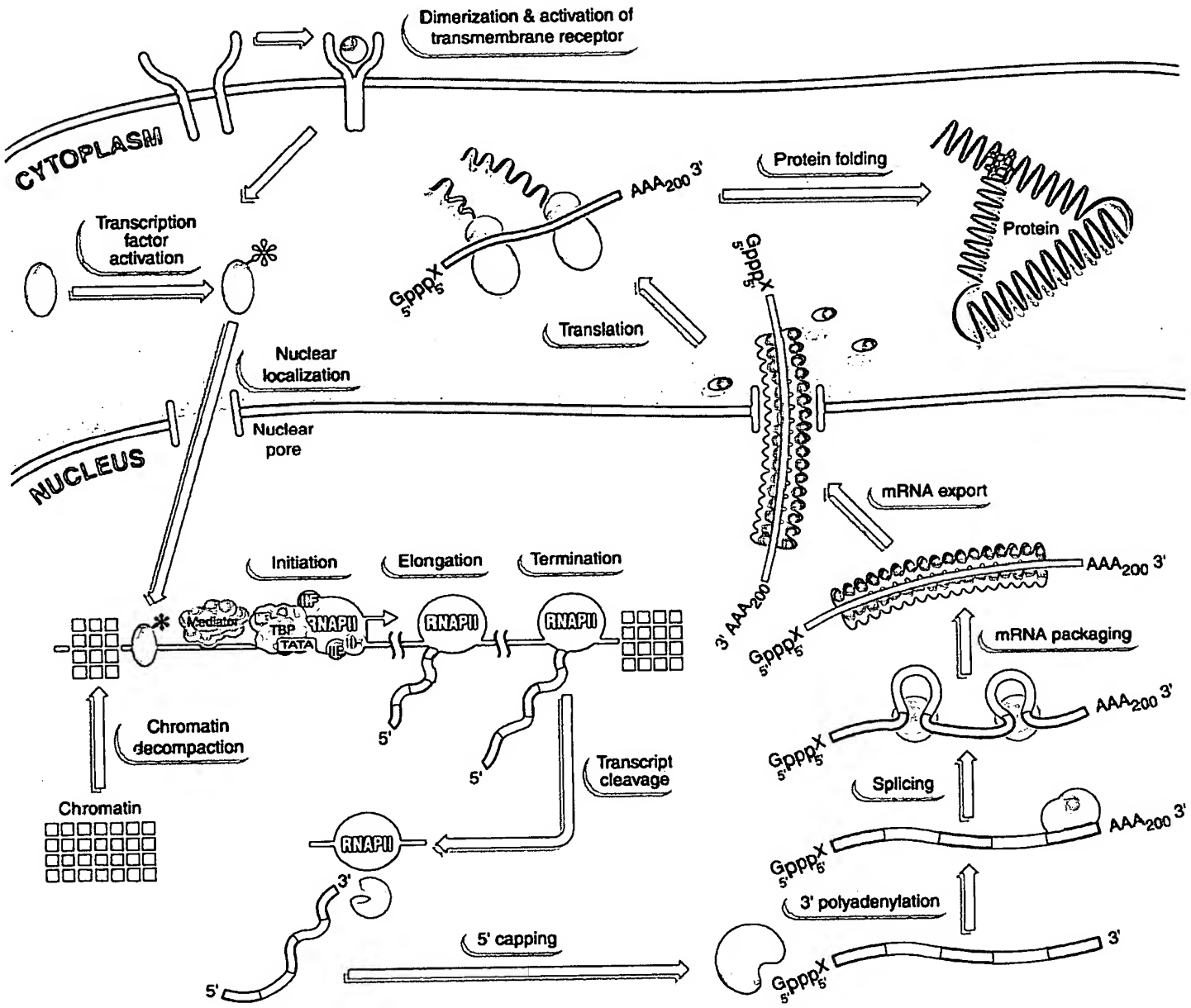
Achillefs Kapanidis (Shimon Weiss' group, UCLA)



Core RNA polymerase (Darst lab)

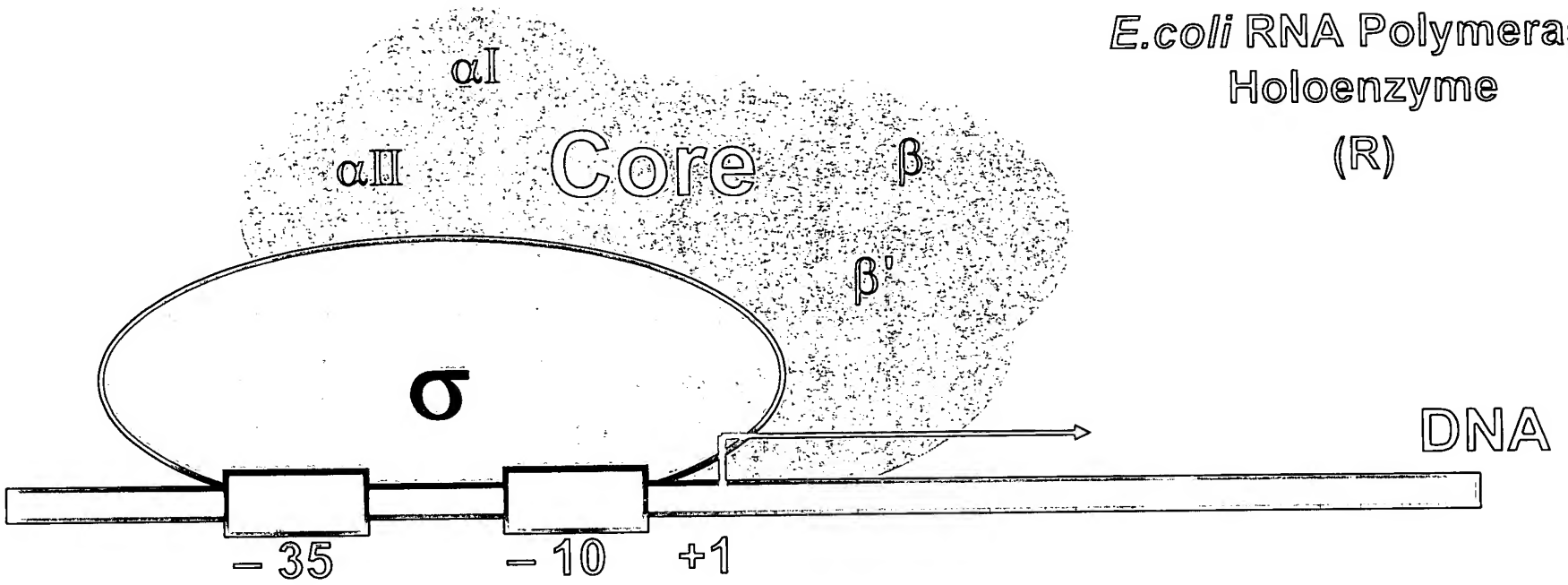
Single-Molecule Biophysics Conference: Aspen, Jan.7, 2003

GENE EXPRESSION:
The path from gene to protein

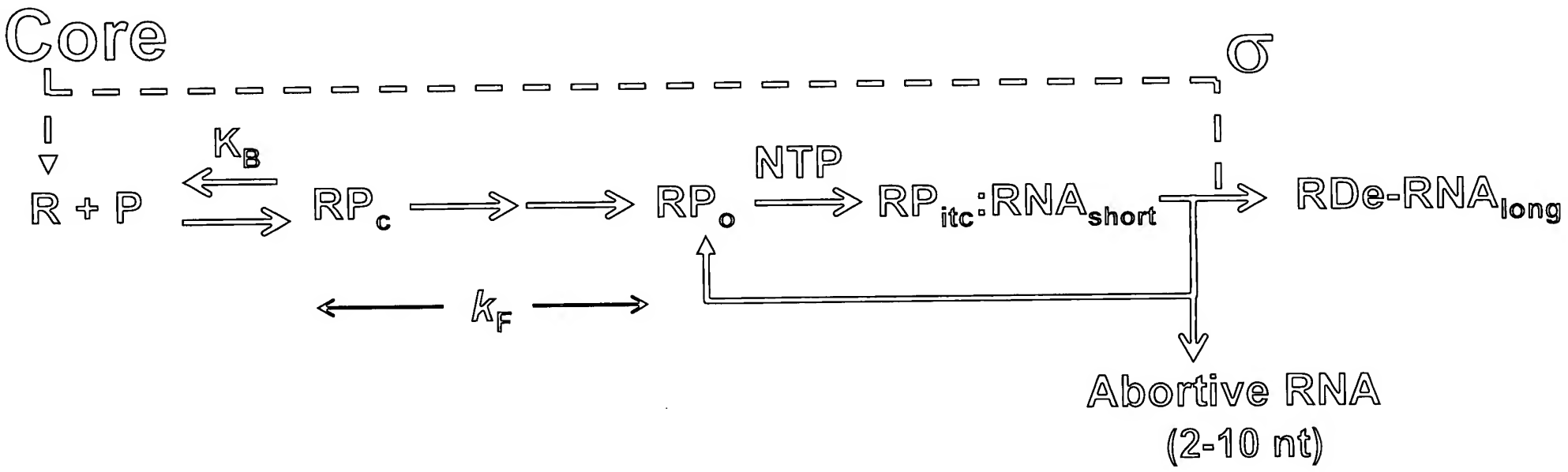


Orphanides and Reinberg, 2002

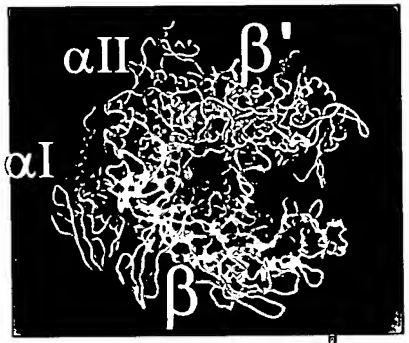
TRANSCRIPTION INITIATION



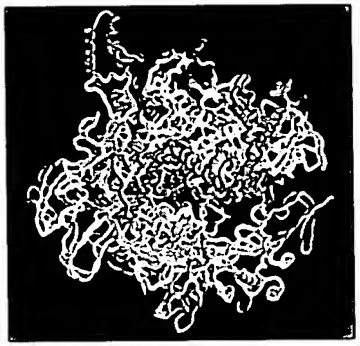
E.coli RNA Polymerase Holoenzyme (R)

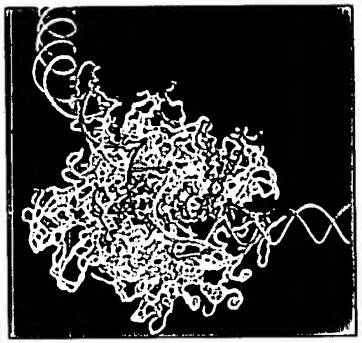


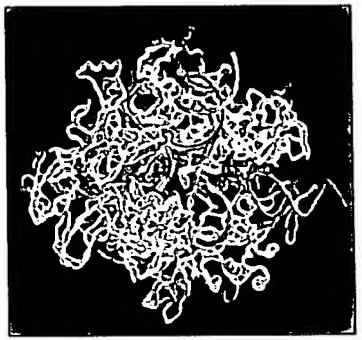
STRUCTURAL ASPECTS OF TRANSCRIPTION



RNAP core ($\alpha_2\beta\beta'$)


RNAP holoenzyme (core + σ)


RP_o


RD_e (model)

X-ray structures → static snapshots of the machine

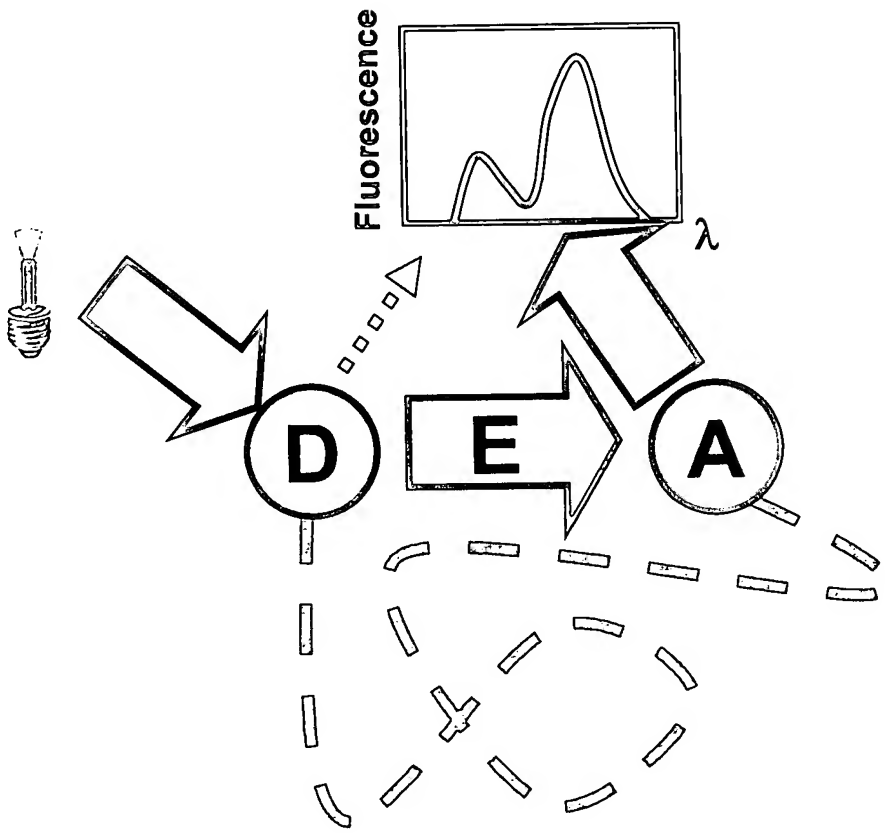
SMD: "movie" of the dynamic process

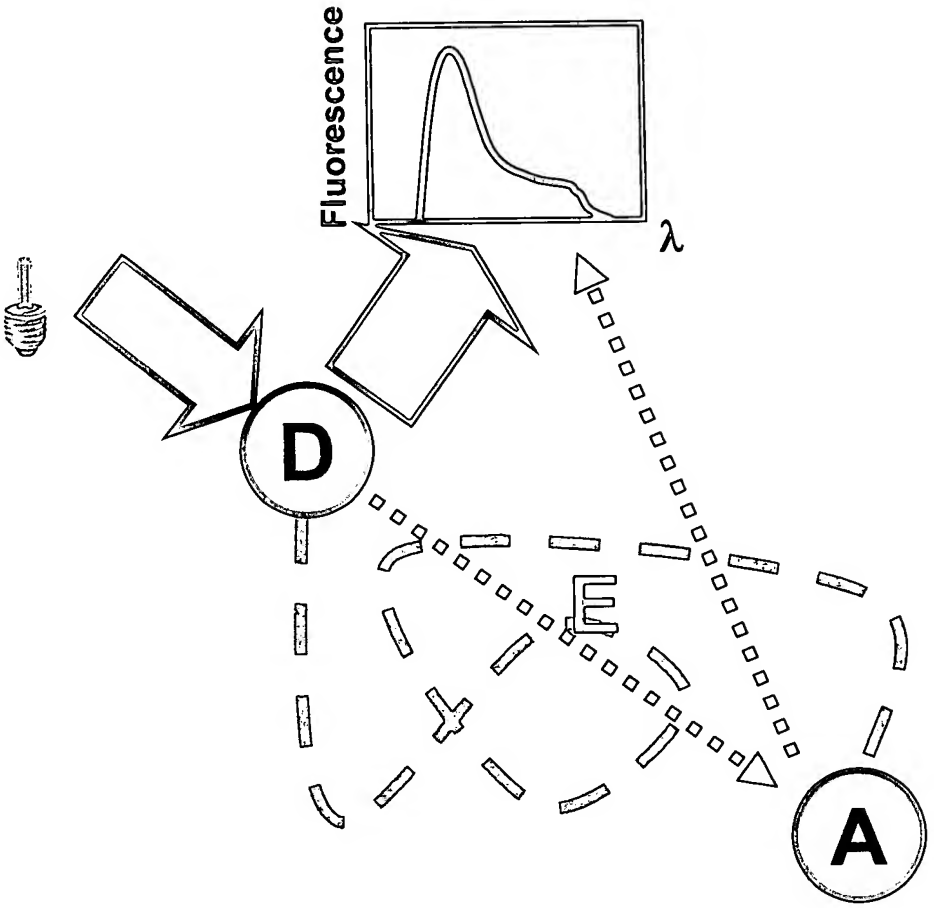
Structure
Dynamics
Local Environment
→ Intermediates
Kinetics
→ Timing
of Events
→ MECHANISM

Young *et al.*, 2002

FÖRSTER RESONANCE ENERGY TRANSFER (FRET):

A "MOLECULAR RULER" FOR THE 2-10 nm REGIME





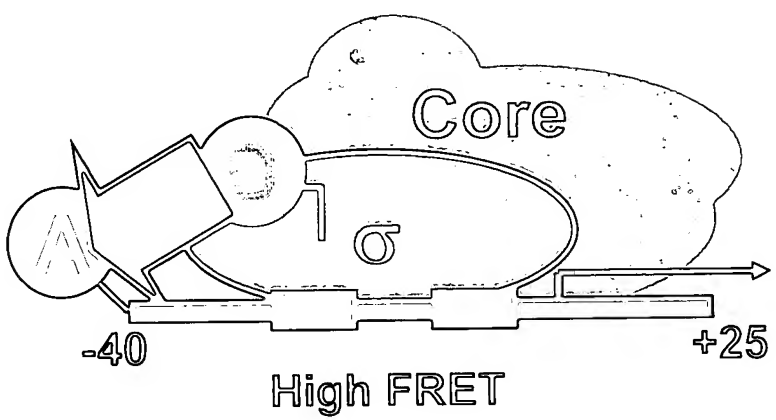
$$\text{FRET Efficiency, } E = [1 + (R/R_o)^6]^{-1}$$

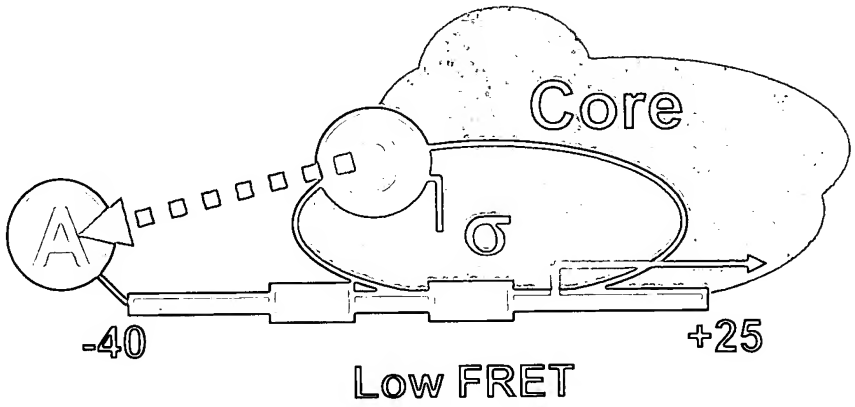
R = D-A Distance

TRAILING-EDGE and LEADING-EDGE FRET:

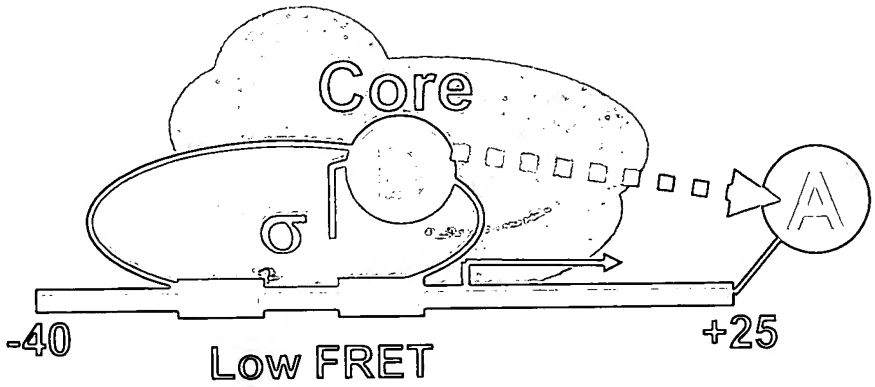
Assay of translocation of a protein relative to a nucleic acid

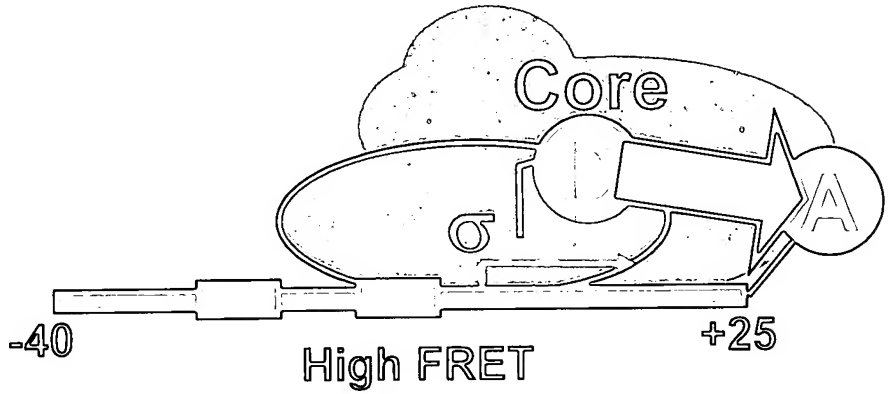
Trailing-edge FRET





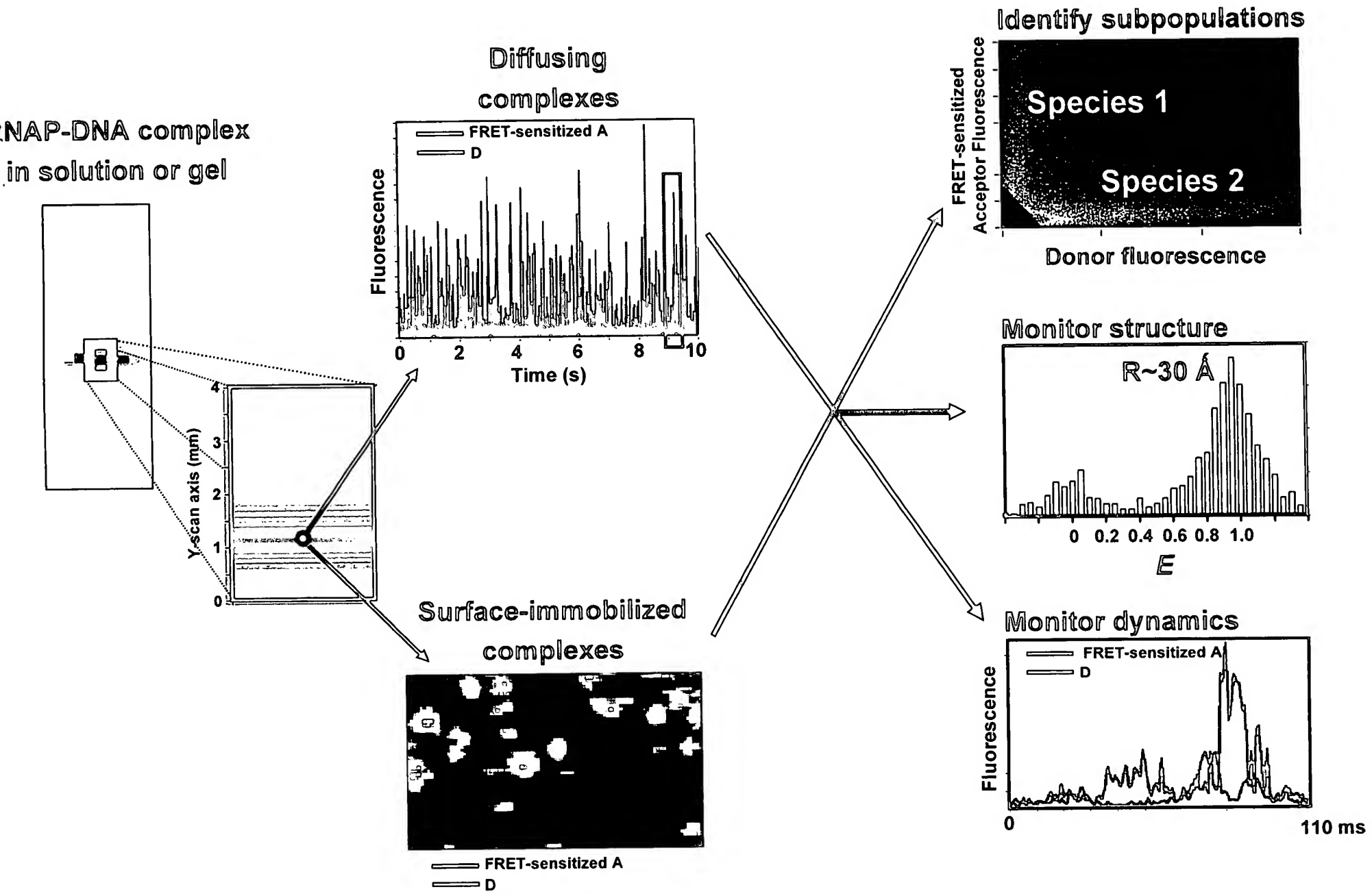
Leading-edge FRET





Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

sp-FRET ON RNAP-DNA COMPLEXES

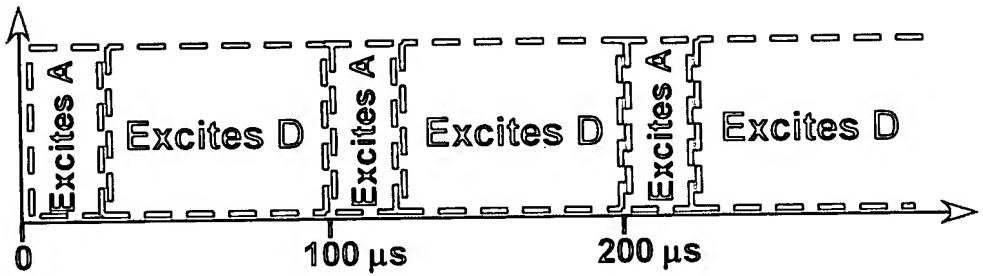


LIMITATIONS OF SINGLE-LASER EXCITATION spFRET

- Complex FRET Acceptor photophysics
  - "Dark" states→D-only peak
  - Photobleaching→ D-only peak
  - Intermittency ("Blinking")
- Complex FRET Donor photophysics
  - Intermittency
  - Transient QY changes
- Limited discrimination ability in the FRET coordinate
  - FRET range of 0-0.3 not easily accessible
- Variable fluorescence contamination
  - Adds variable counts to D-only peak

sp-FRET USING ALTERNATE LASER EXCITATION (ALEX)

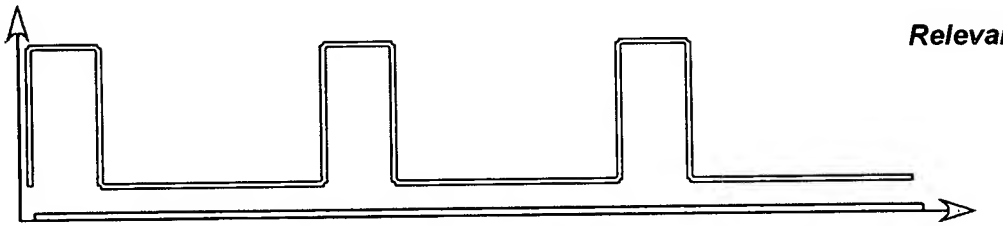
EXCITATION PROFILE



EMISSION PROFILES

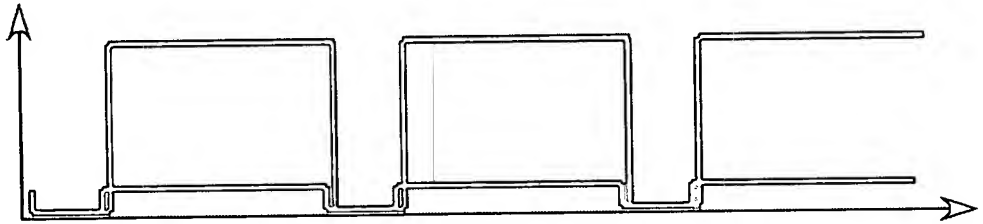
Relevant transcription species

A-only species



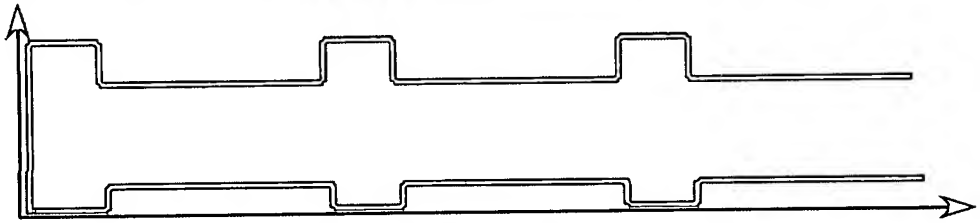
DNA^{A}

D-only species



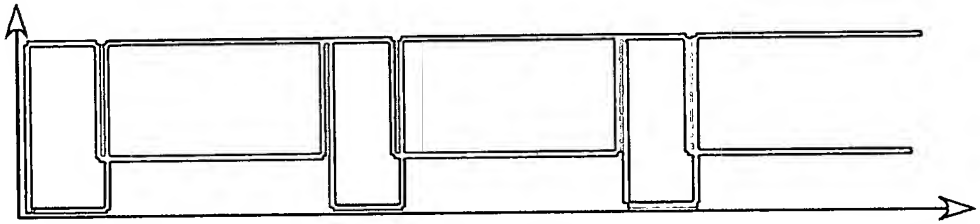
$RNAP\sigma^{D}$

D-A species, high FRET



RPo^{D-A}, Trailing-edge

D-A species low FRET



RPo^{D-A}, Leading-edge

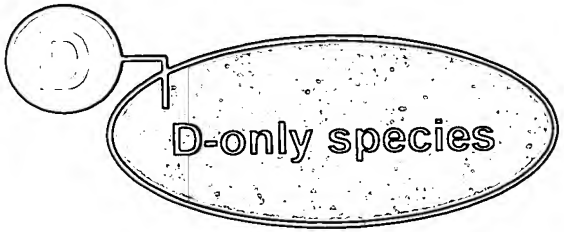
EQUATIONS

Energy transfer ratio (*E*)

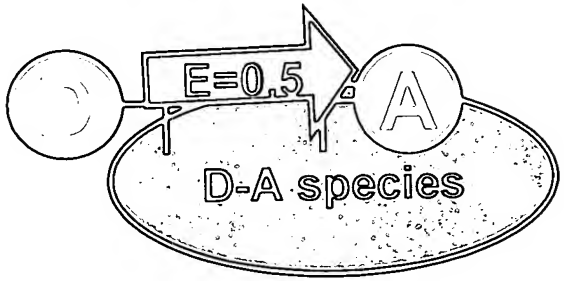
$$E = \frac{F^{DA}_{670em,514ex}}{F^{DA}_{670em,514ex} + F^{DA}_{580em,514ex}}$$

ALEX-based ratio (*ALEX*)

$$ALEX = \frac{F_{514ex}}{F_{514ex} + F_{638ex}} = \frac{F_{670em,514ex} + F_{580em,514ex}}{F_{670em,514ex} + F_{580em,514ex} + F_{670em,633ex}}$$



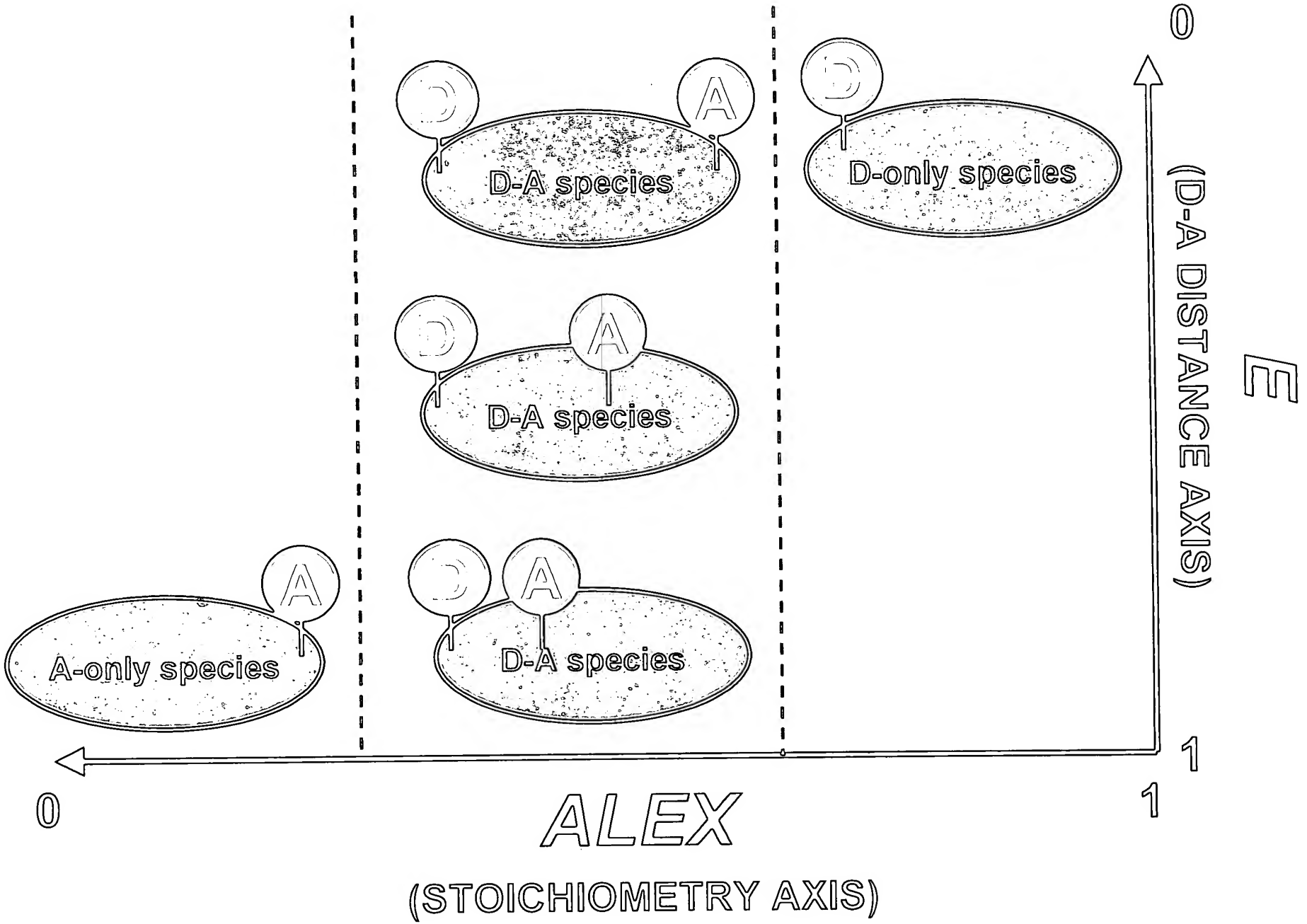
$$ALEX = \frac{0+100}{0+100+0} \sim 1.0$$



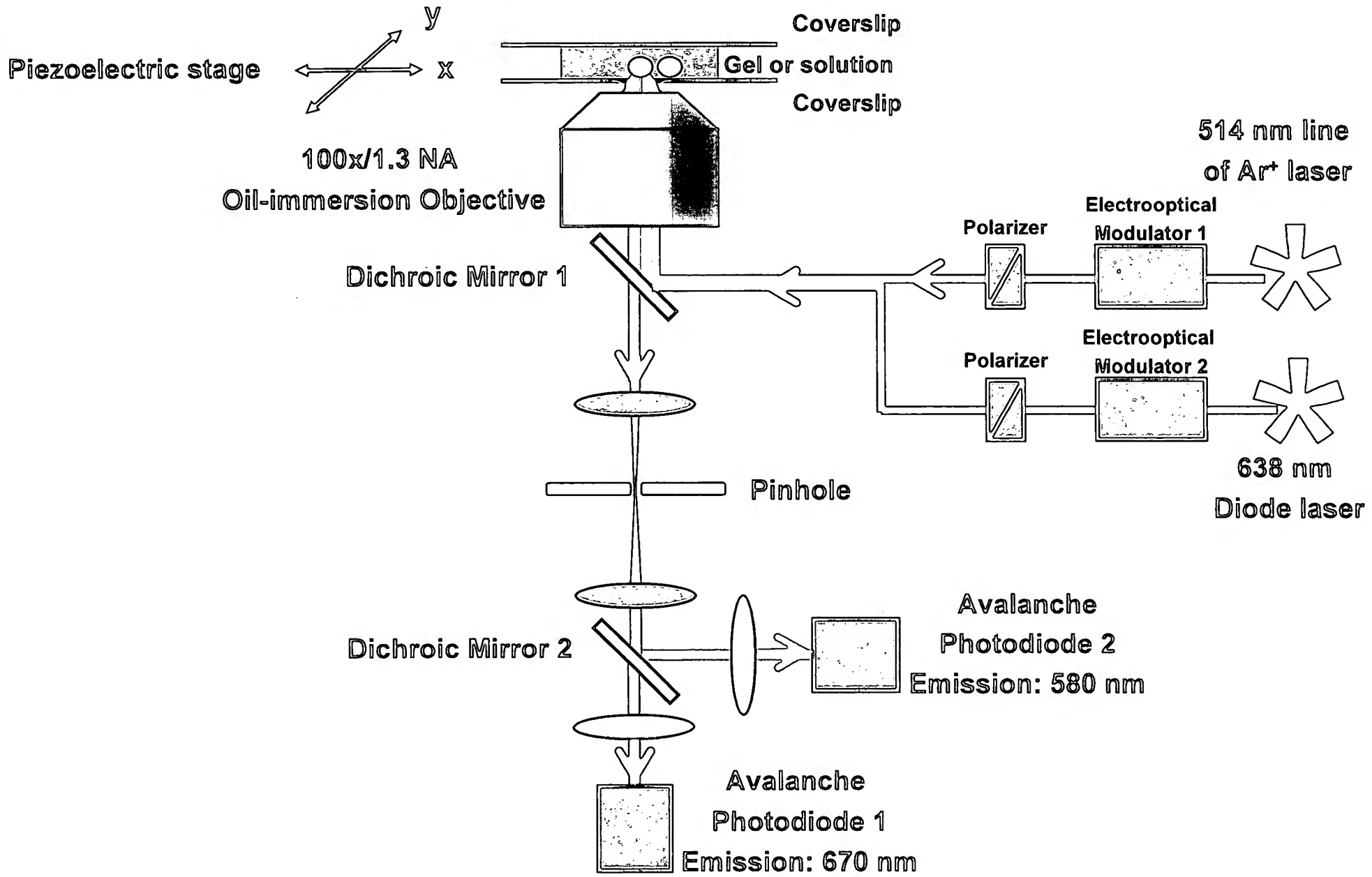
$$ALEX = \frac{50+50}{50+50+100} \sim 0.5$$

$$ALEX = \frac{0+0}{0+0+100} \sim 0.0$$

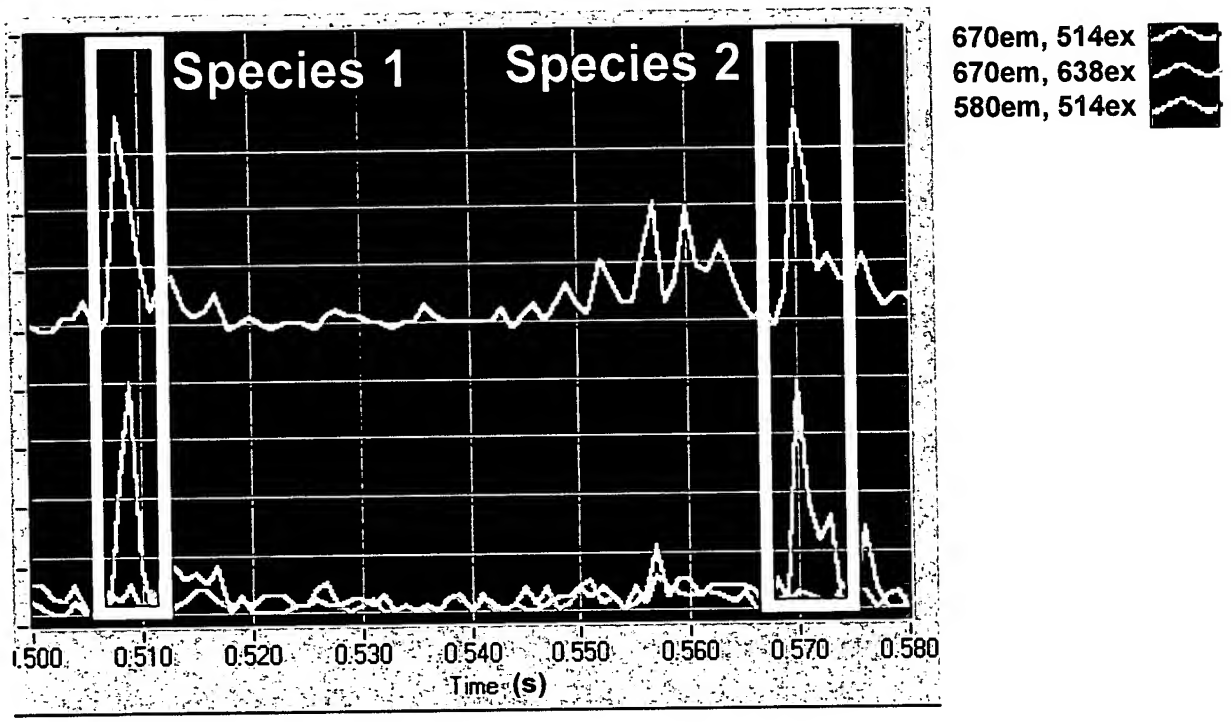
SORTING SPECIES USING *E*, *ALEX*



ALEX SINGLE-MOLECULE CONFOCAL MICROSCOPY

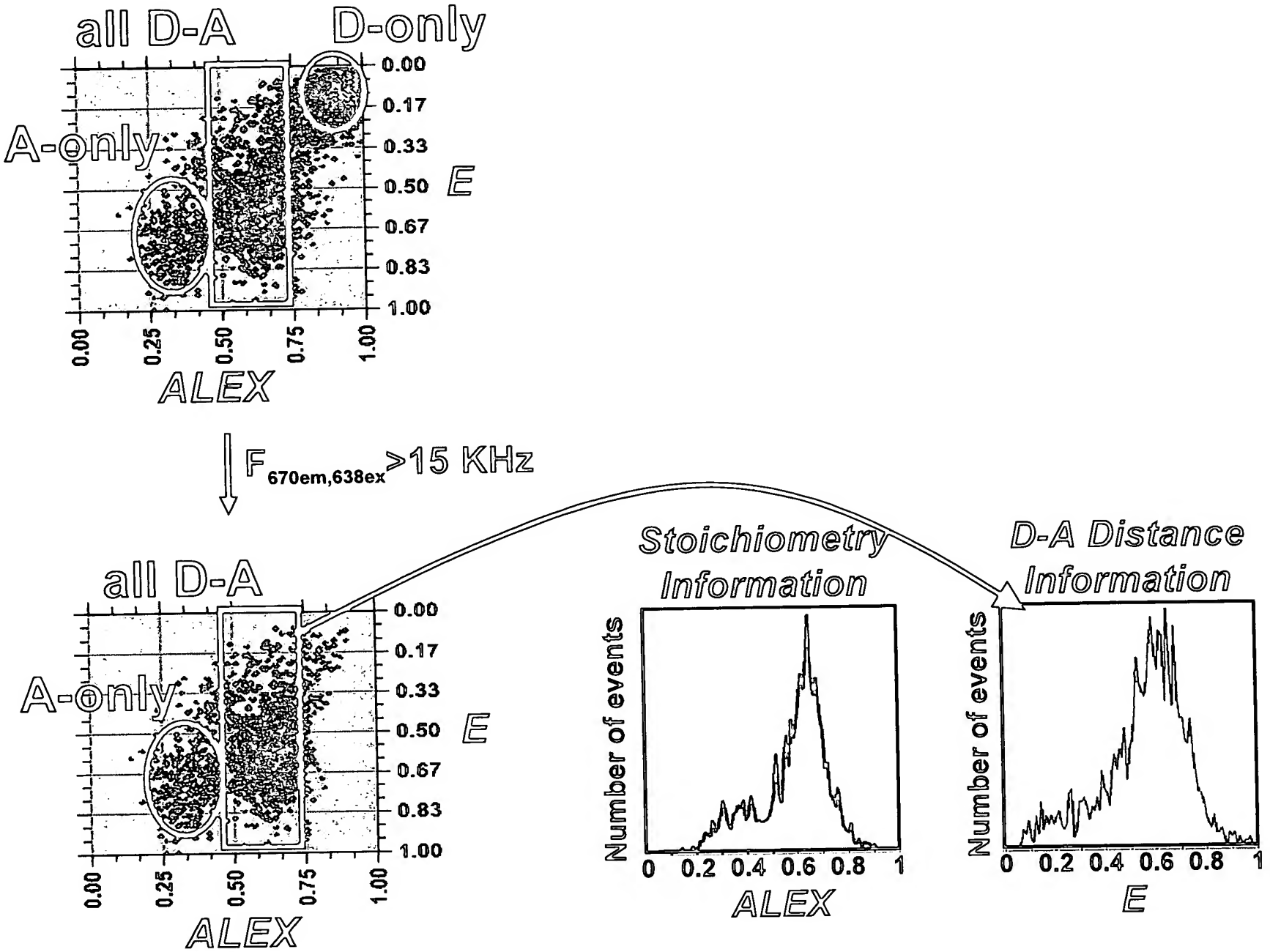


DATA ANALYSIS FOR INDIVIDUAL SPECIES

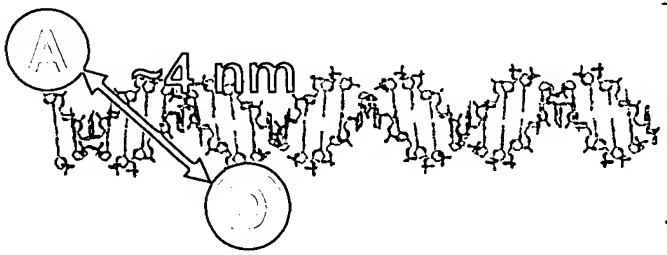


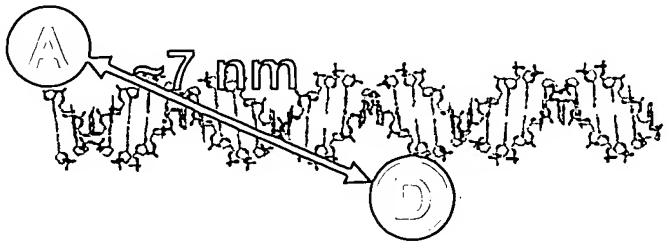
	Species 1	Species 2
670em, 514ex	71	85
670em, 638ex	69	93
580em, 514ex	7	11
FRET-sensitized A	52	60
E, simplified	91%	88%
E, FRET-sensitized A	91%	77%
ALEX	0.49	0.66

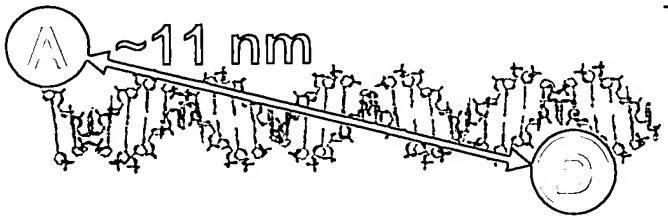
DATA ANALYSIS USING *E-ALEX* 2-D HISTOGRAMS



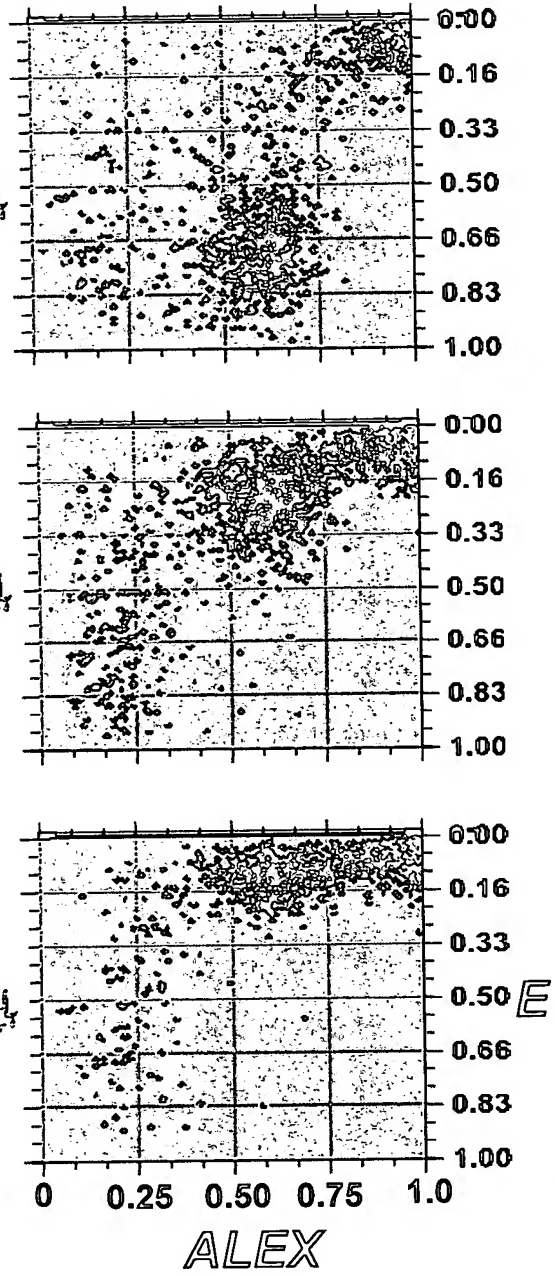
MODEL SYSTEMS: dsDNA

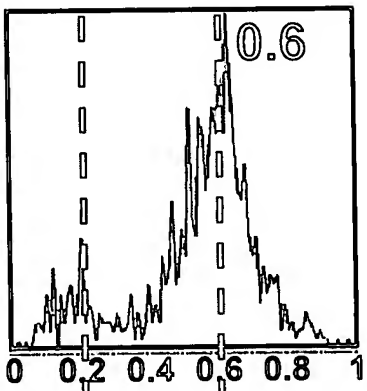


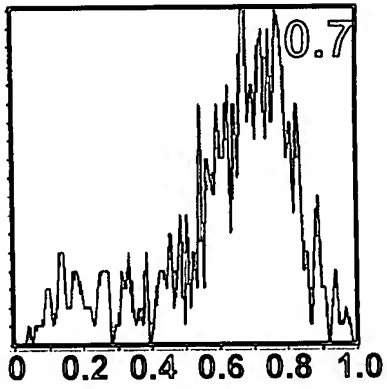


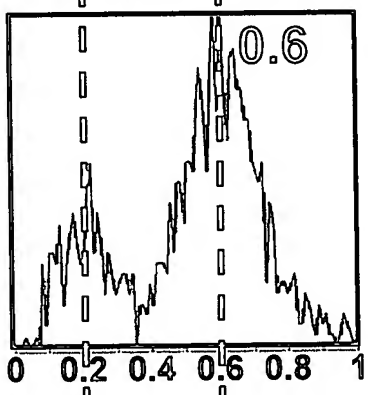


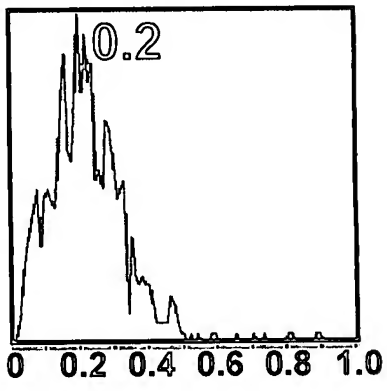
$R_{o,D-A}$~5.5 nm

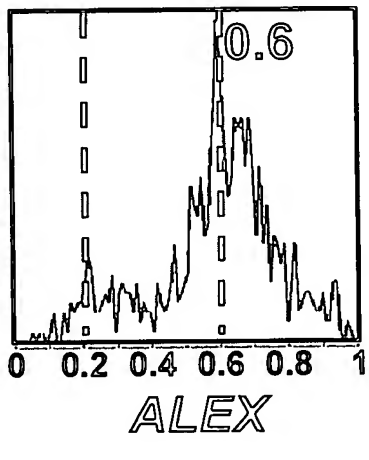


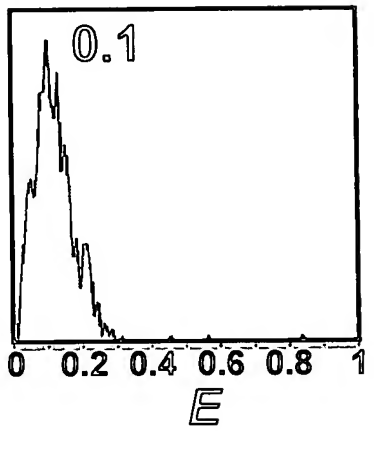






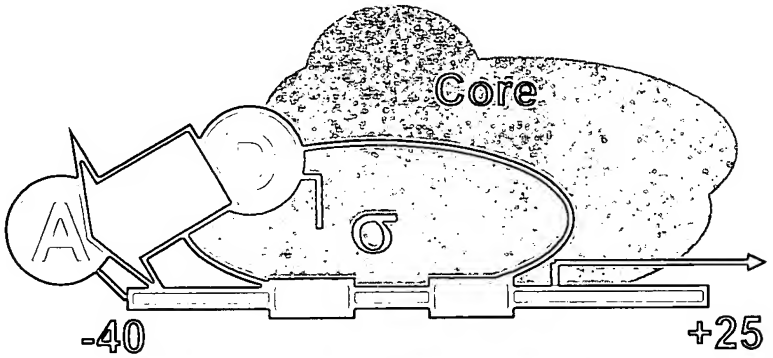






USING TRAILING-EDGE sp-FRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE

OPEN COMPLEX

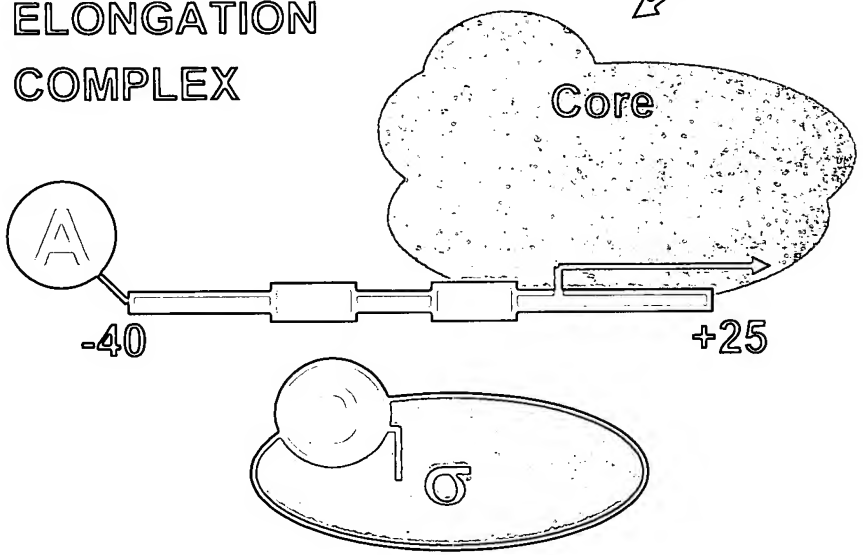


D and A co-localize; High E

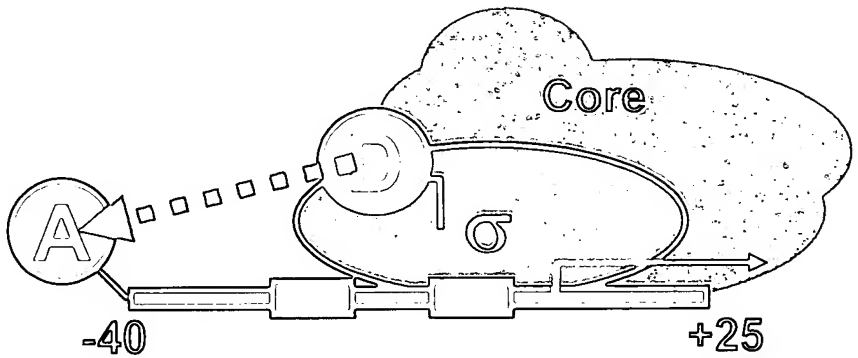
σ release model

σ non-release model

ELONGATION COMPLEX



D and A do *not* co-localize; Zero E



D and A co-localize; Zero or low E

Mukhopadhyay *et al.*, 2001

TRAILING-EDGE spFRET

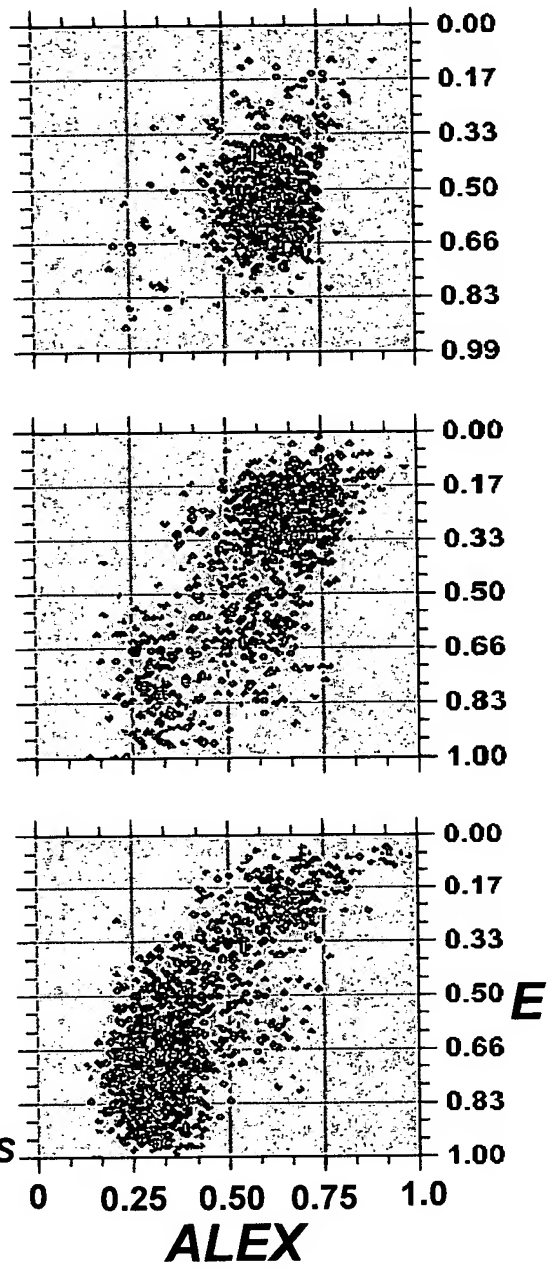
RNAP$\sigma^{TMR,569}$→lacUV5-11$^{Cy5,-40}$

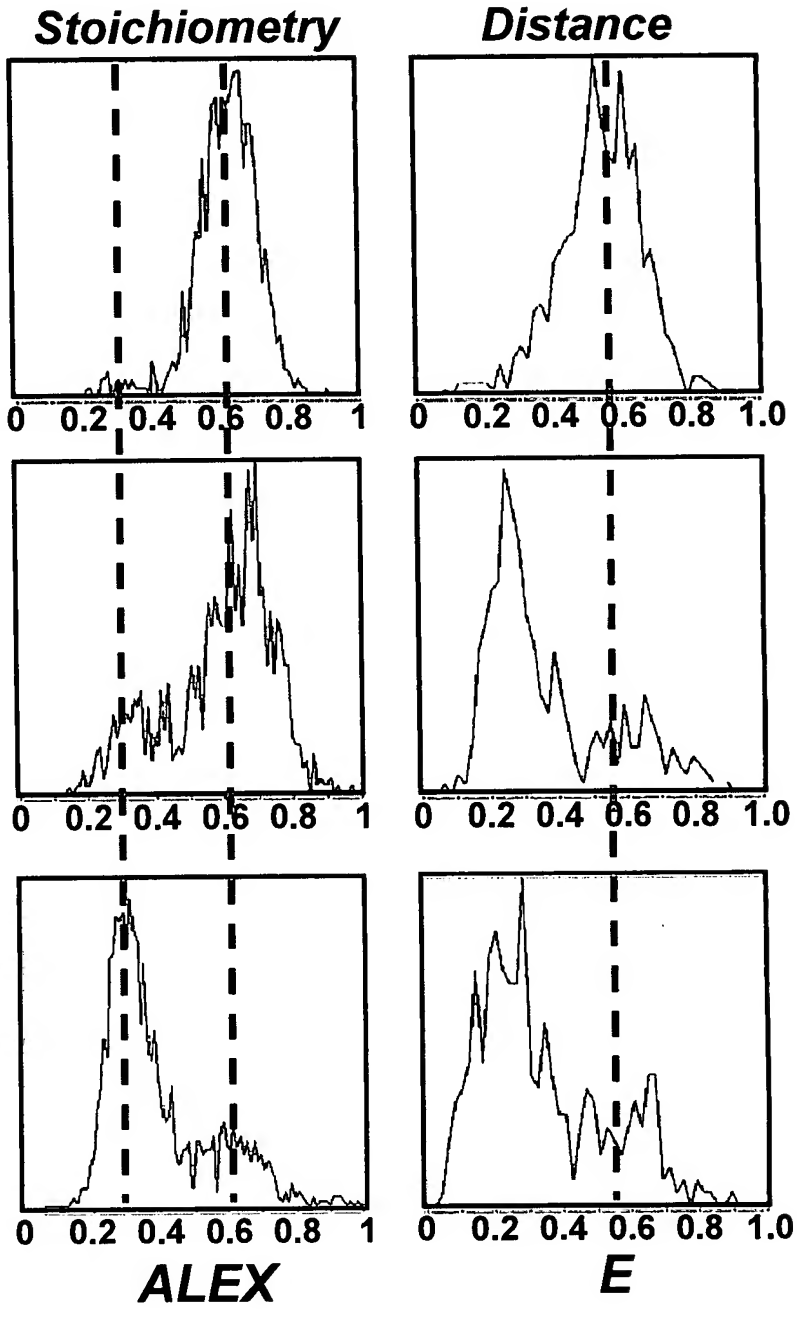
RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

RPo + ApA
+ 60 μM NTPs
(chase)

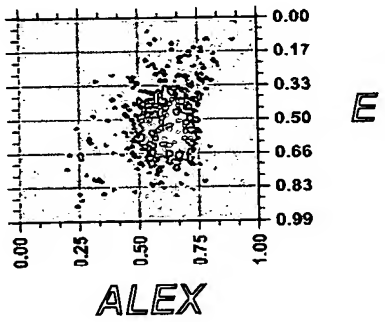
ABILITY OF STALLED COMPLEXES TO RESUME TRANSCRIPTION

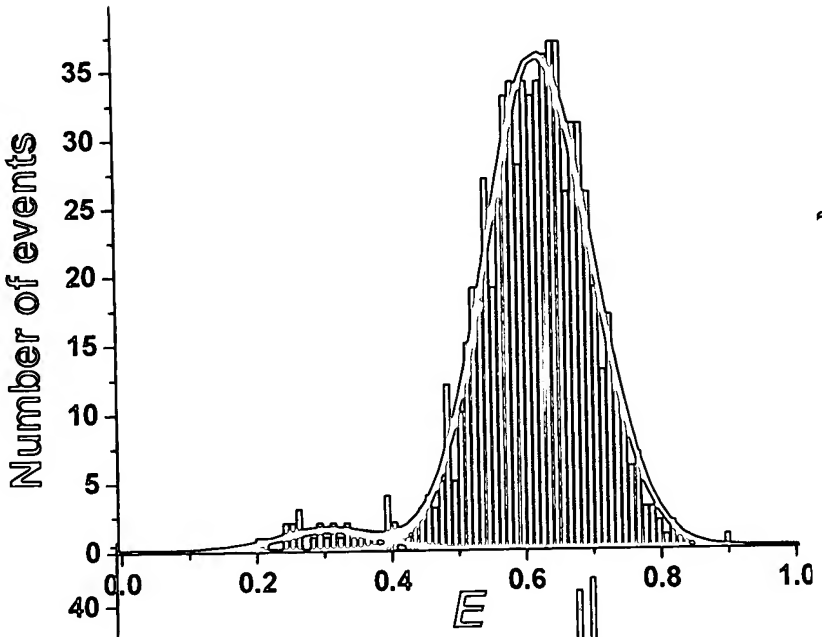




DIRECT OBSERVATION OF SIGMA NON-RELEASE: TRAILING-EDGE spFRET

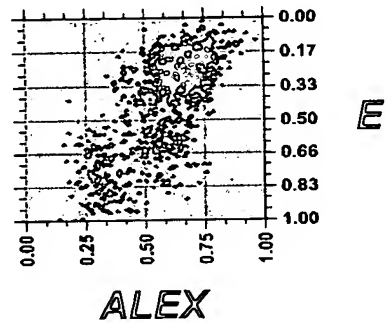
$RP_{itc,2}$

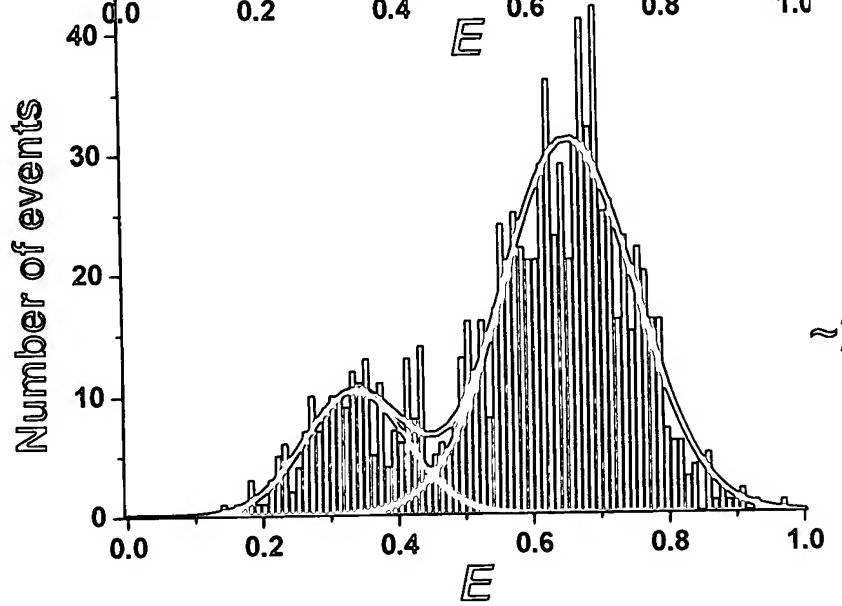




~5% dissociation

$RD_{e,11}$



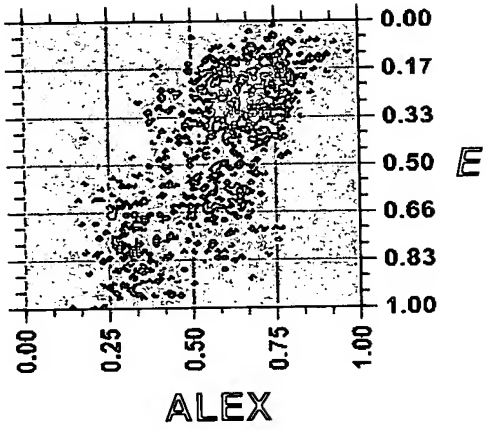


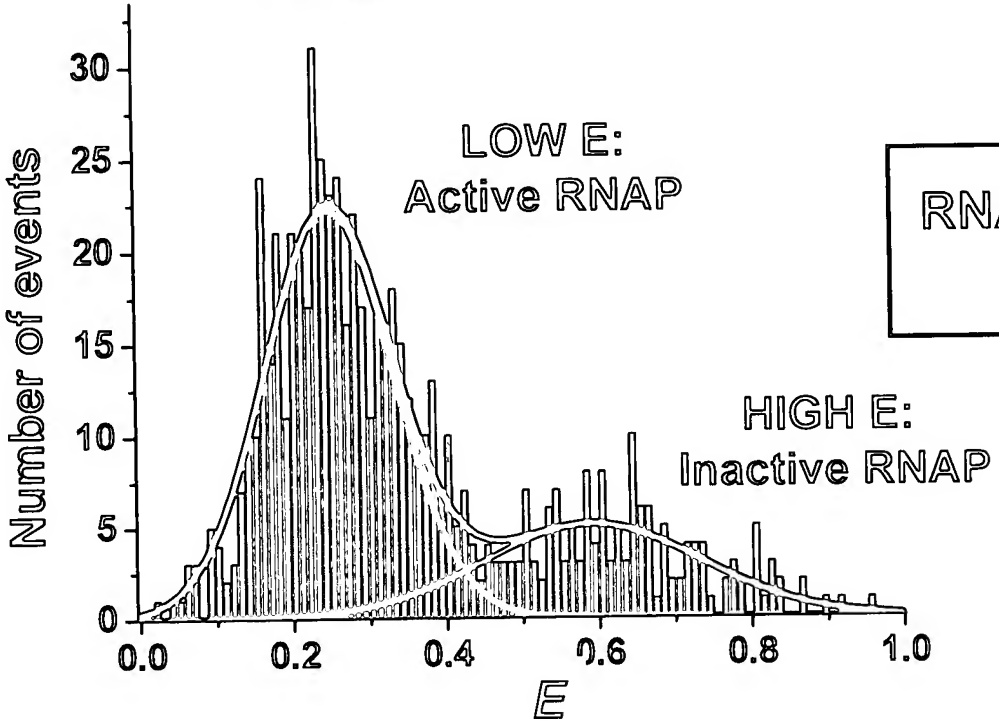
~20% dissociation

$$\% \text{ Dissociation} = \frac{(\text{A-only})}{(\text{A-only})+(\text{all D-A})}$$

E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: TRAILING-EDGE spFRET

RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

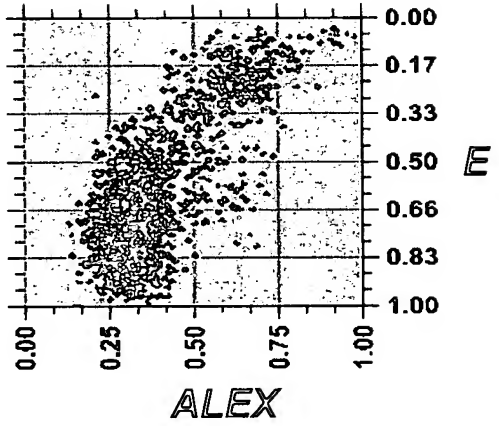


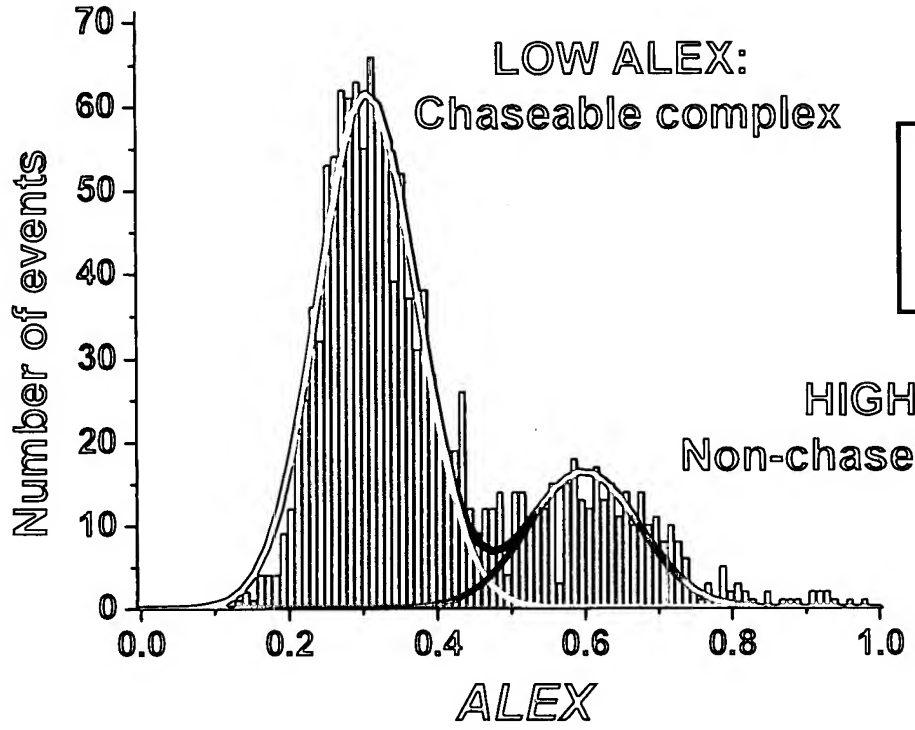


RNAP translocational activity ~ 70%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": TRAILING-EDGE spFRET

RPo + ApA + 60 μM NTPs (chase)

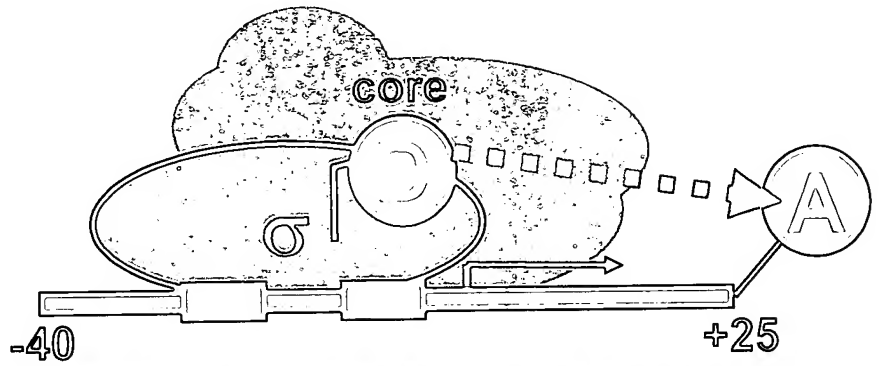




RNAP "chaseability"
~ 80%

USING LEADING-EDGE spFRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE

OPEN COMPLEX

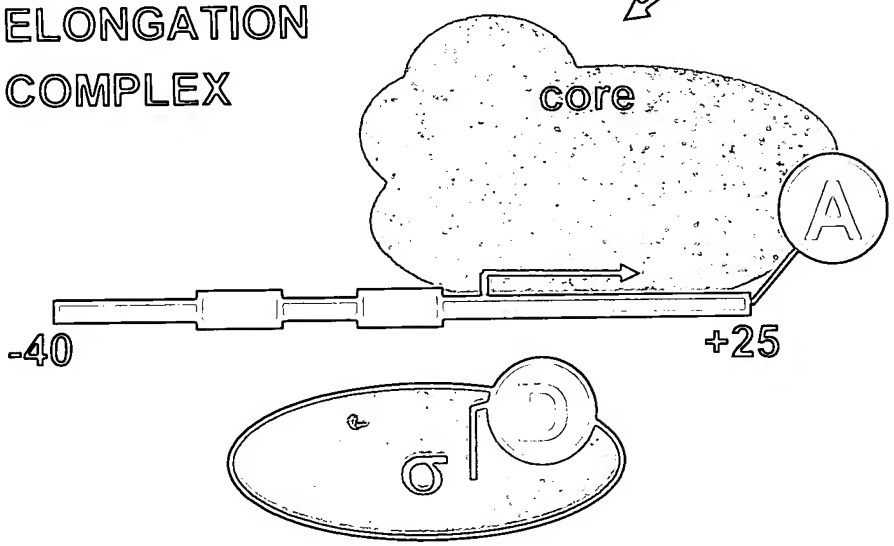


D and A co-localize; Low or zero E

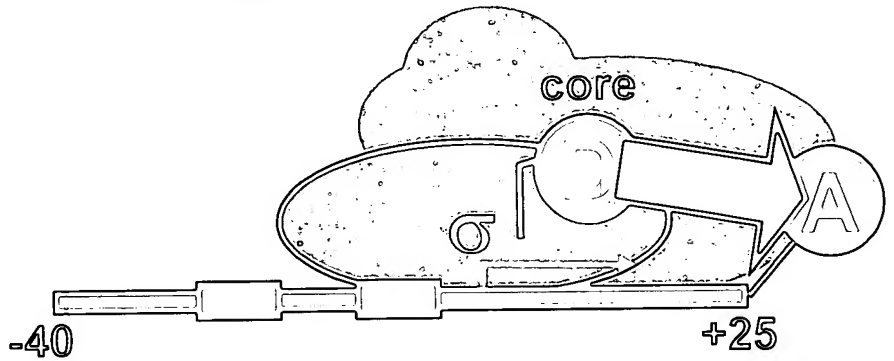
σ release model

σ non-release model

ELONGATION COMPLEX



D and A do *not* co-localize; Zero E

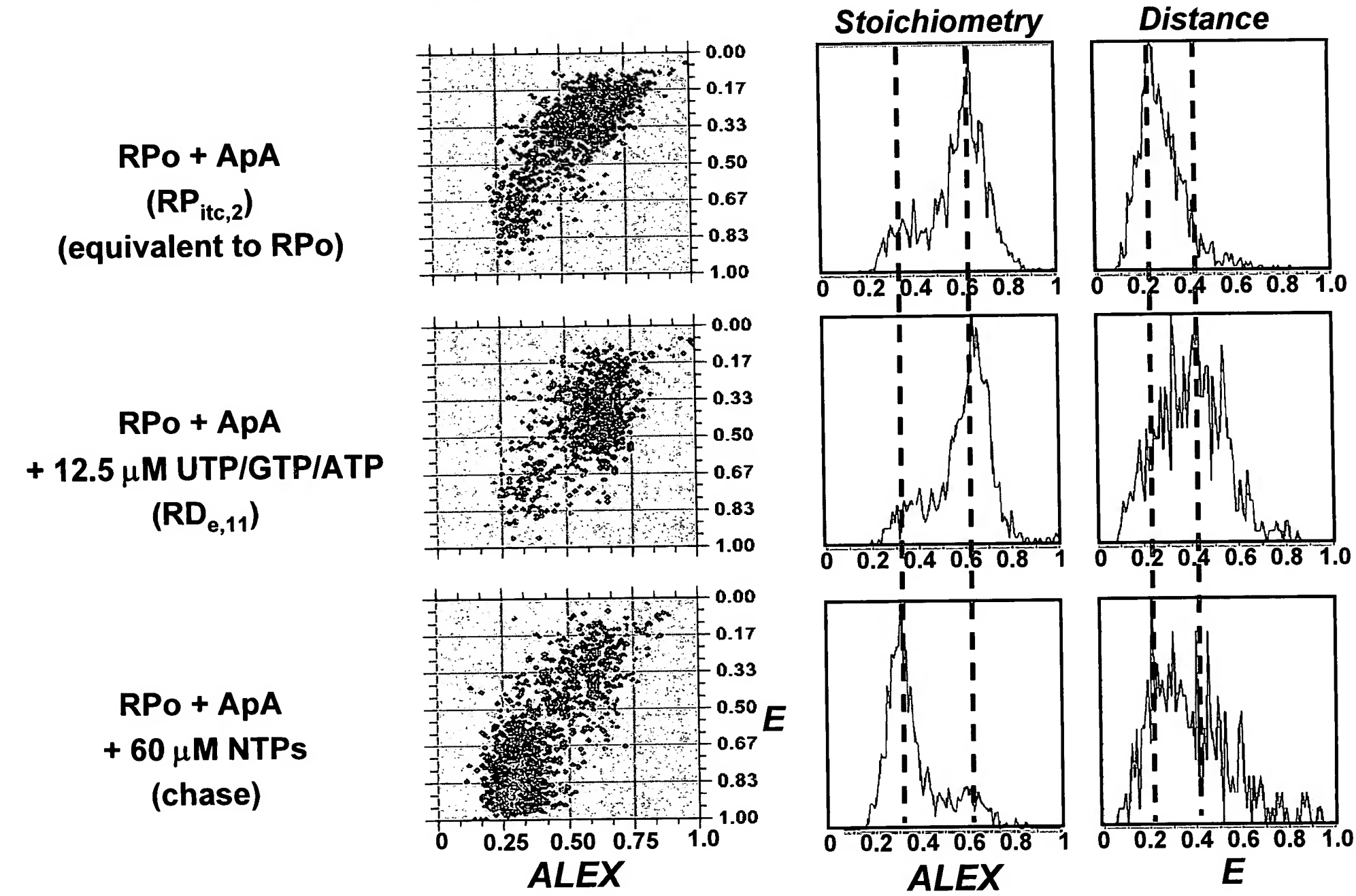


D and A co-localize; High E

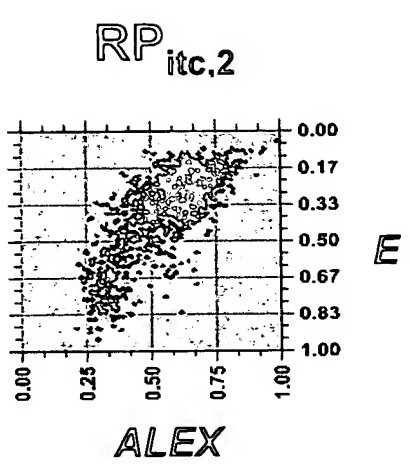
Mukhopadhyay *et al.*, 2001

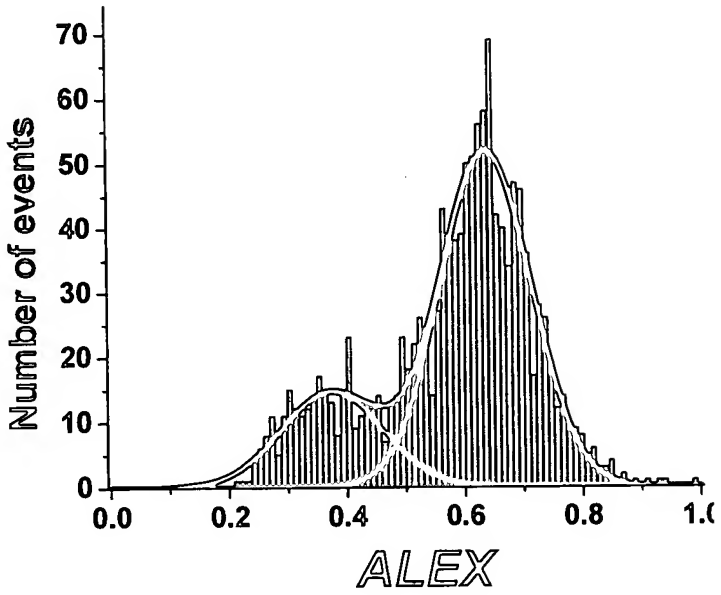
LEADING-EDGE spFRET

$RNAP\sigma^{TMR,366} \rightarrow lacUV5\text{-}11^{Cy5,+25}$

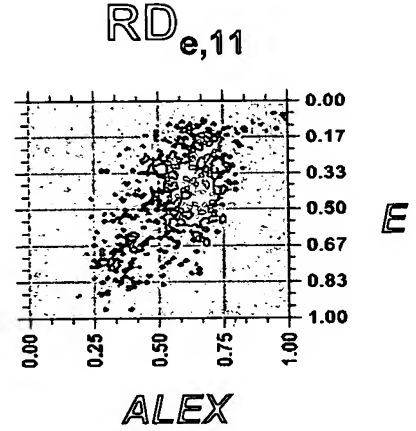


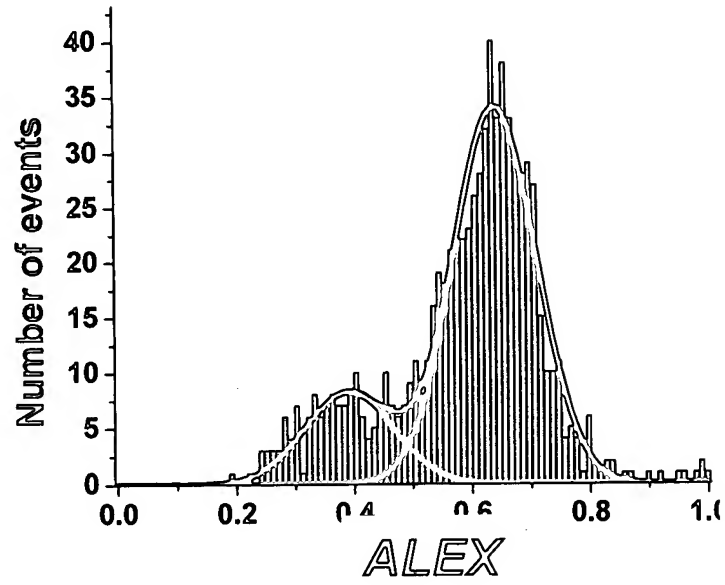
DIRECT OBSERVATION OF SIGMA NON-RELEASE: LEADING-EDGE spFRET





~20% dissociation

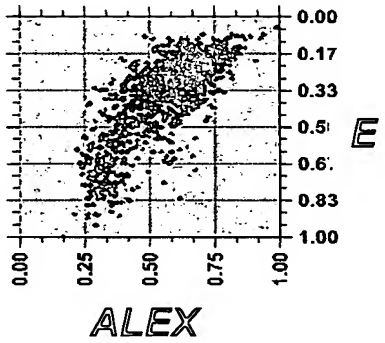


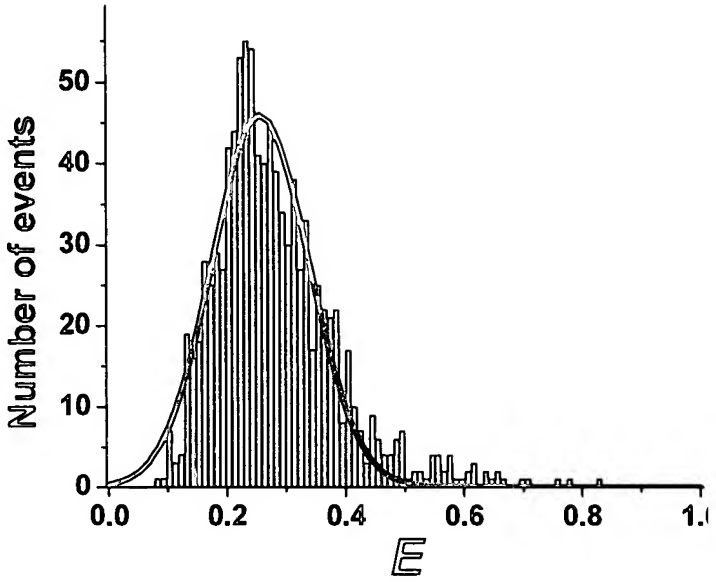


~20% dissociation

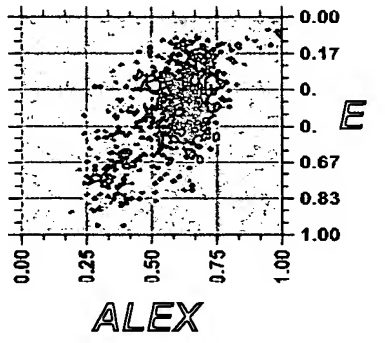
E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: LEADING-EDGE spFRET

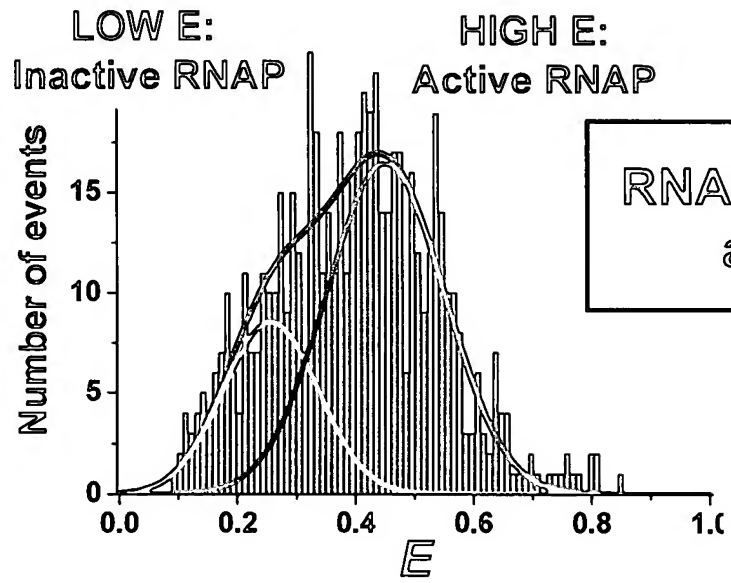
RPo + ApA ($RP_{itc,2}$)





RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

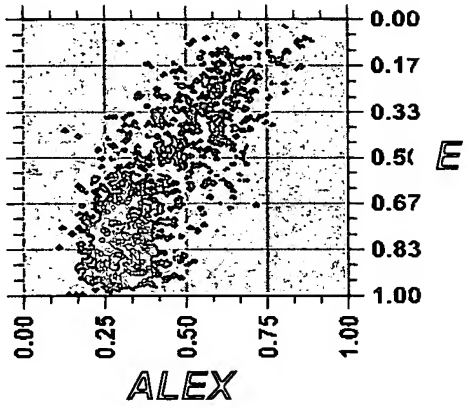


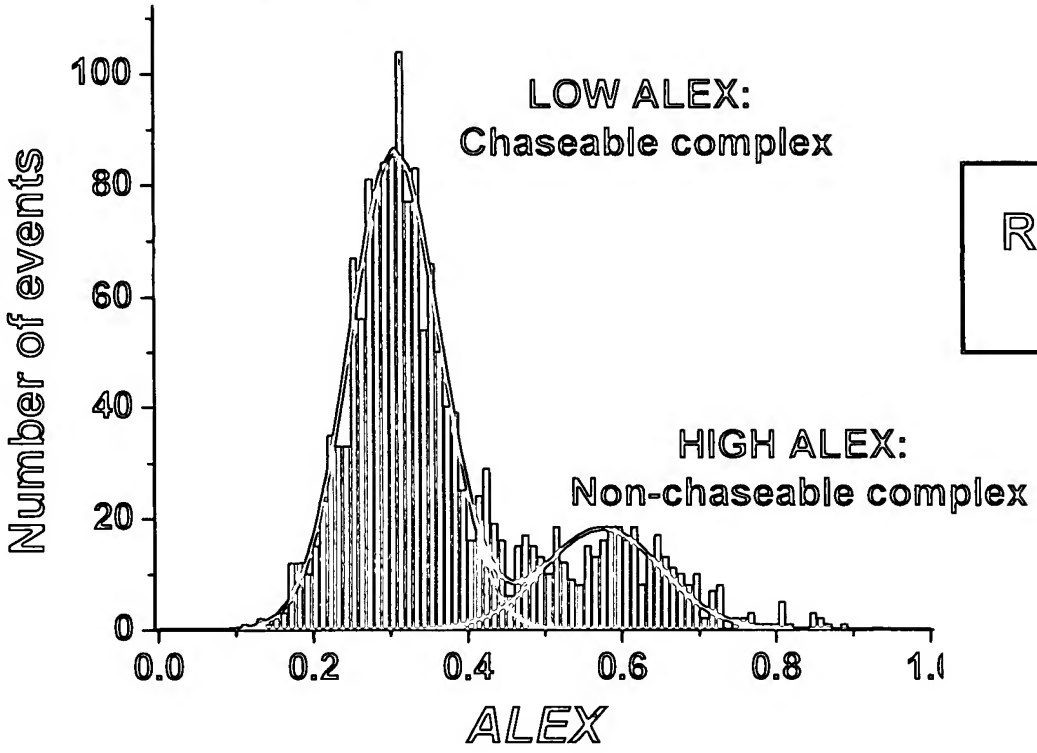


RNAP translocational activity = 72%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": LEADING-EDGE spFRET

RPo + ApA + 60 μM NTPs (chase)

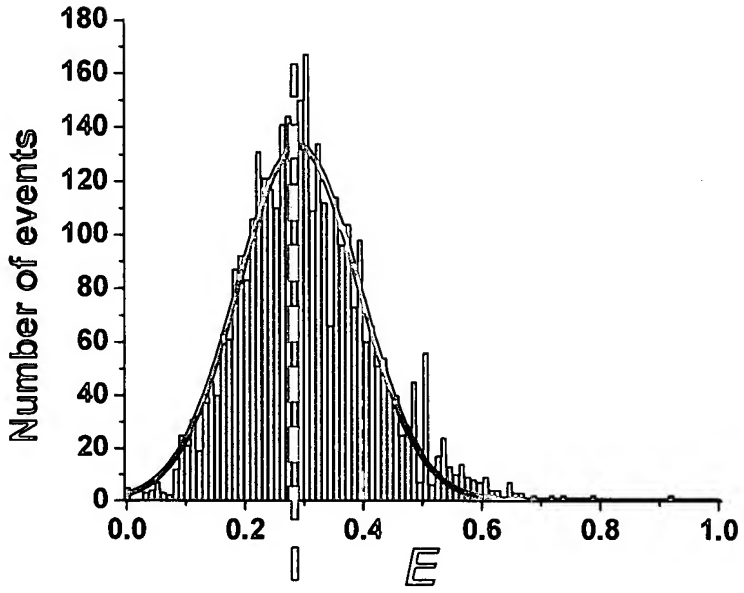




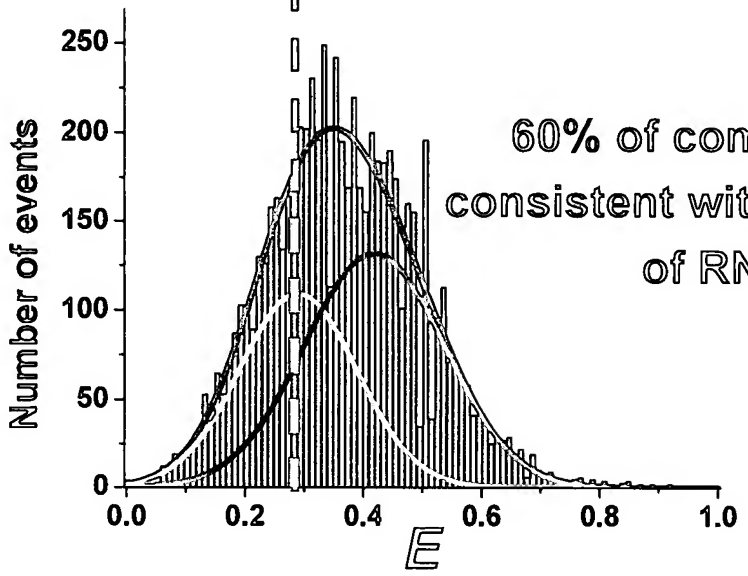
RNAP "chaseability" = 80%

LEADING-EDGE spFRET DETECTS MOVEMENT OF LEADING EDGE DURING ABORTIVE INITIATION

RPo + ApA
($RP_{itc,2}$)



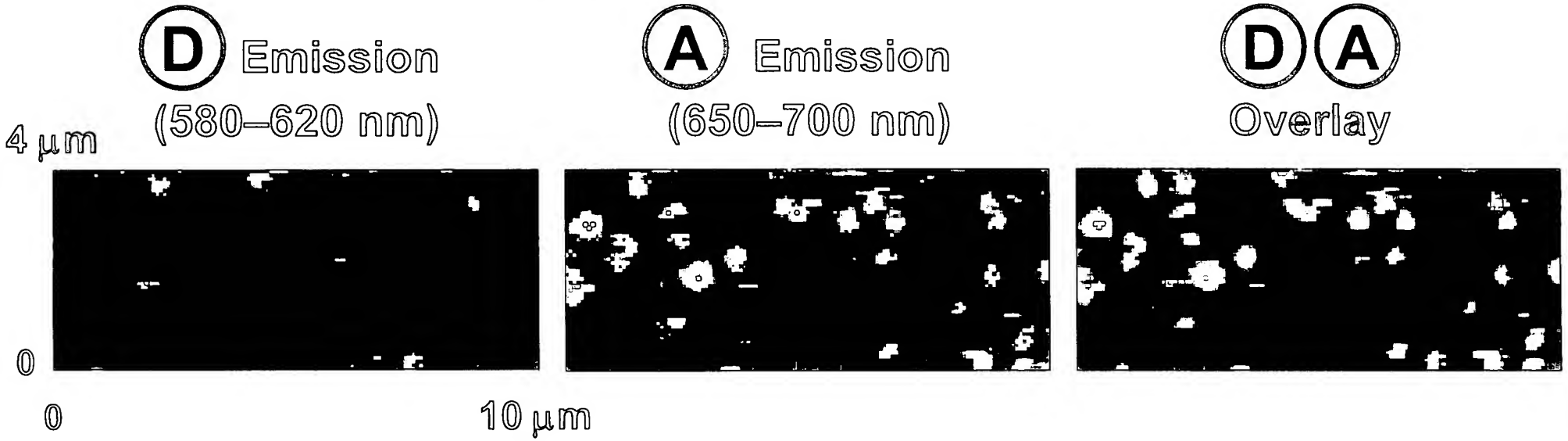
RPo + ApA
+ 25 μM UTP/GTP
($RD_{e,7}$)



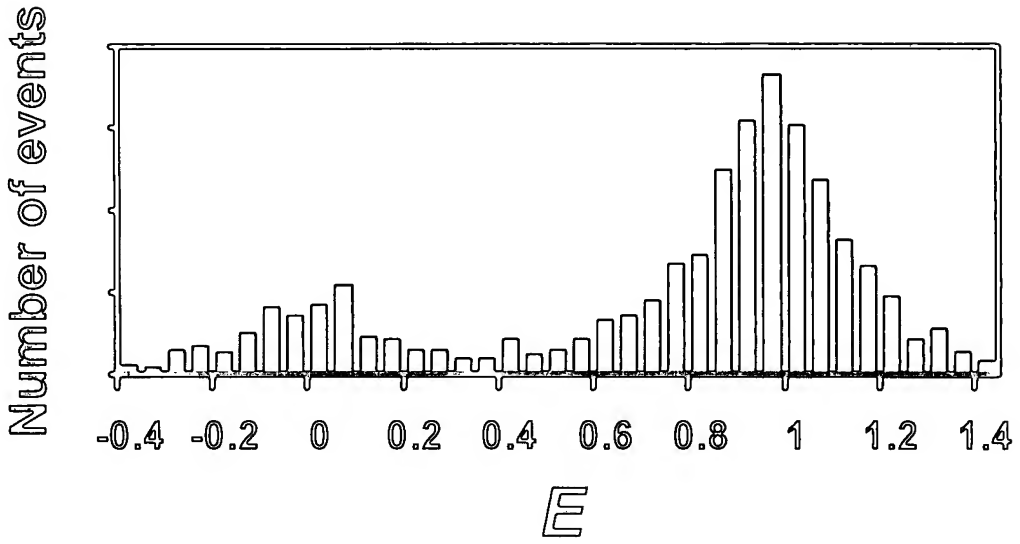
60% of complexes show higher *E*; consistent with downstream movement of RNAP leading edge

TRAILING-EDGE spFRET ON SURFACE-IMMOBILIZED RP_o COMPLEXES

Excitation: 514 nm line of Ar^+ laser

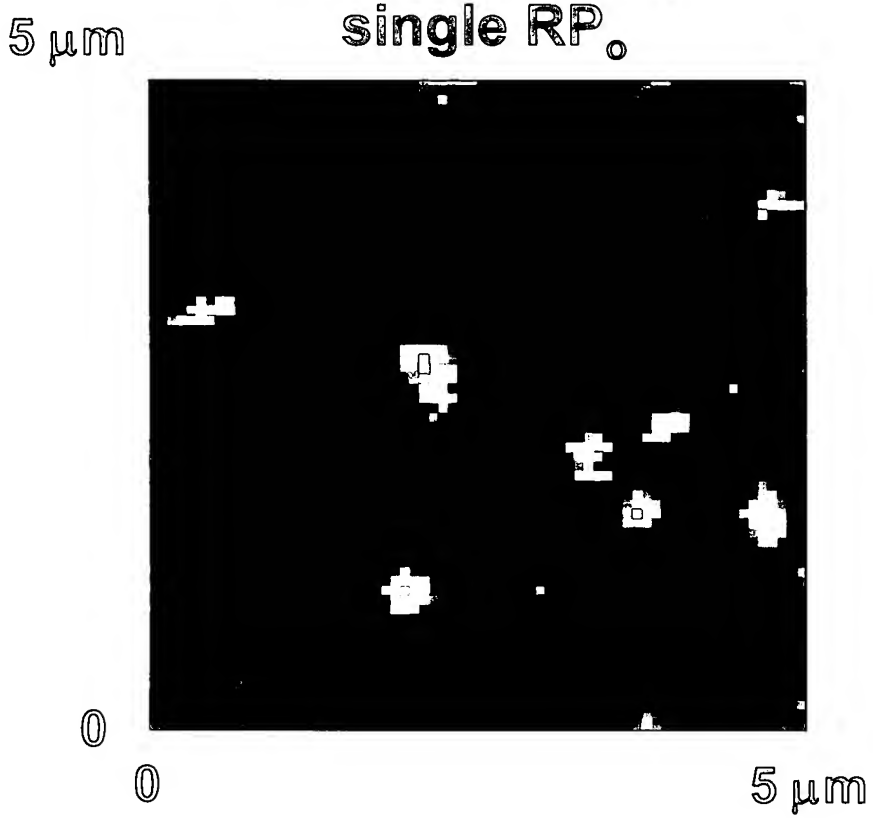


$$E = \frac{I_A}{I_A + \gamma I_D}$$

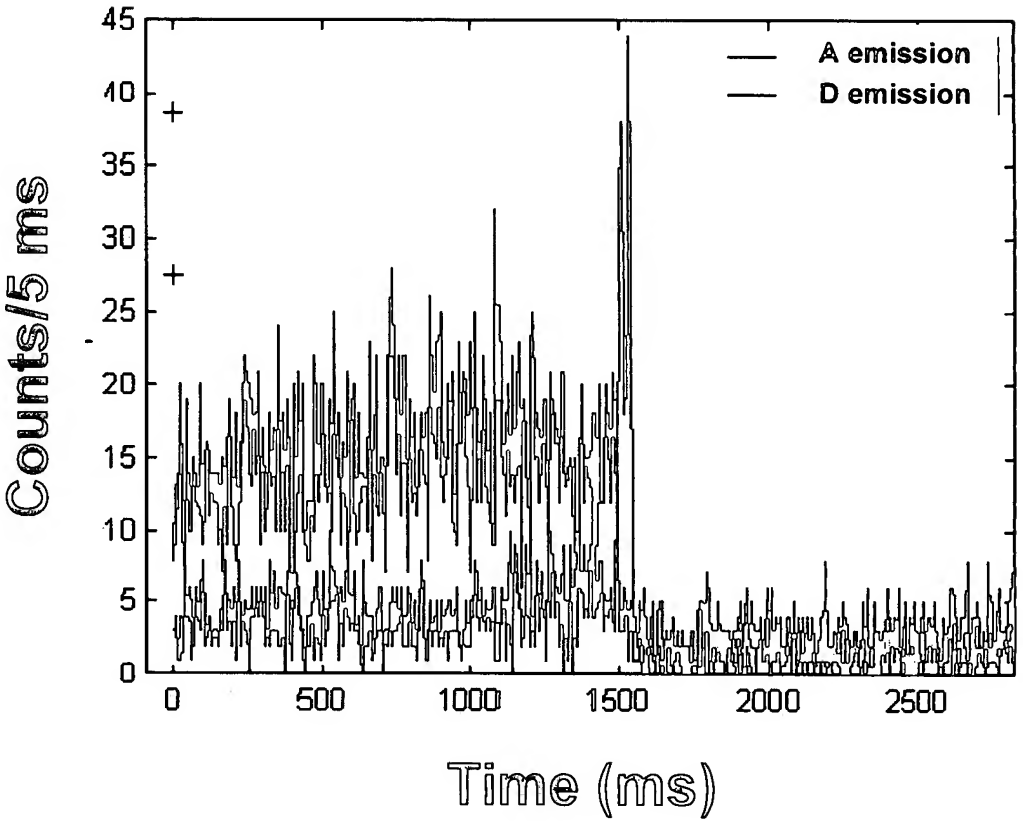


IMAGING AND TIME-TRAJECTORIES OF SINGLE RP_o COMPLEXES

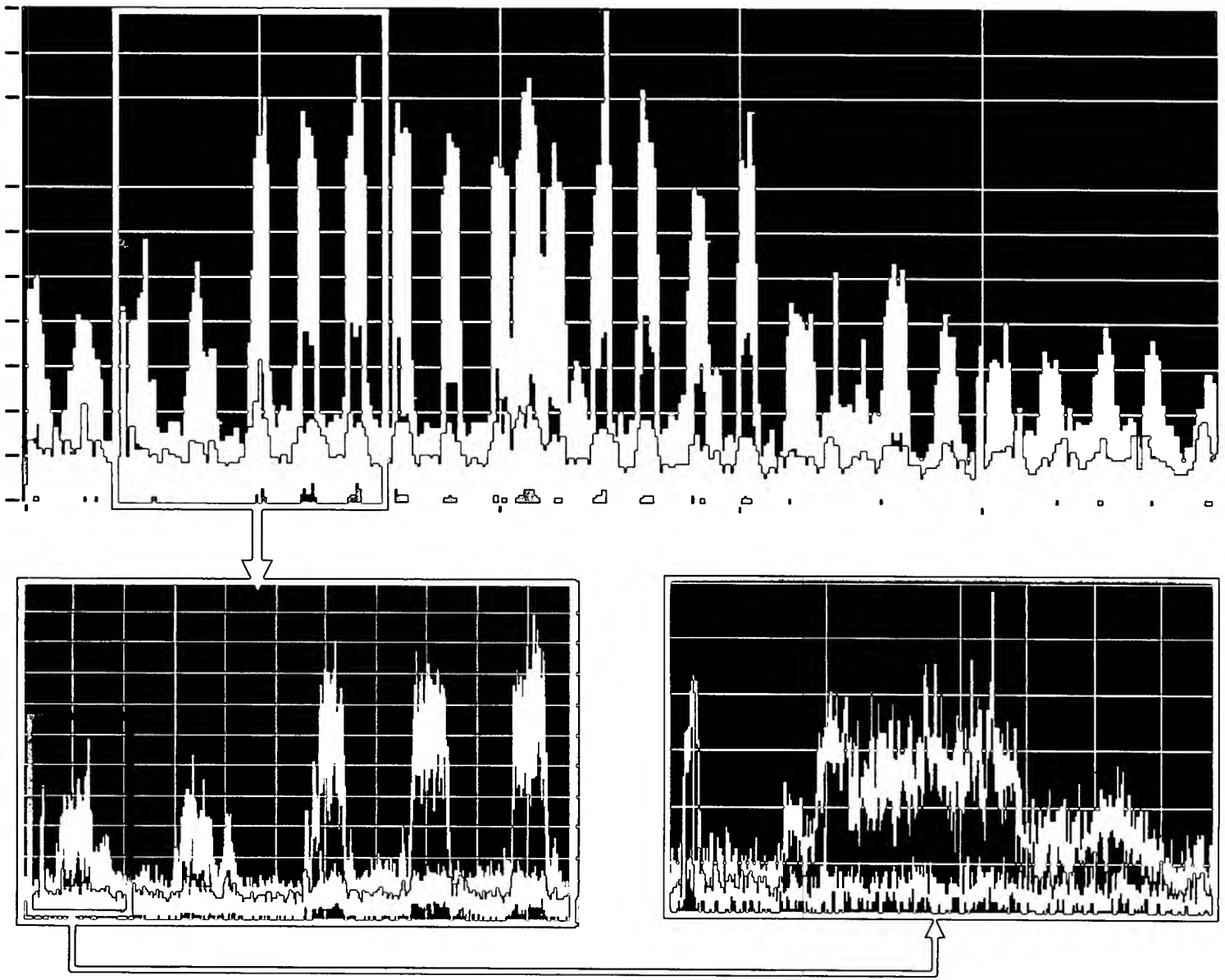
Single-step photobleaching: evidence for imaging single RP_o



Time-trajectory for a single RP_o showing TE-FRET



MONITORING SINGLE-ENZYME DYNAMICS ON IMMOBILIZED MOLECULES



CONCLUSIONS

- Developed robust assays for analysis of structure, dynamics, and activity of protein-DNA complexes
- Confirmed sigma presence in early elongation complexes
- Determined activity for translocation and for chase reactions
- Detected movement of leading edge during abortive initiation
- Future work:
    - Abortive initiation mechanism
    - Sigma dynamics at various transcription steps

ACKNOWLEDGEMENTS

Shimon Weiss (UCLA)
Sören Doose
Thilo Lacoste
Ted Laurence
Nam Ki Lee
Emmanuel Margeat
Xavier Michalet

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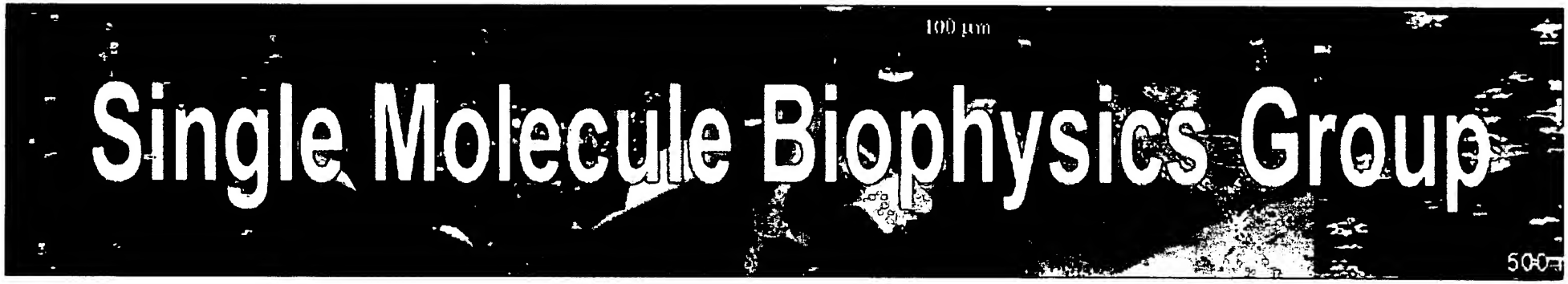
Richard Ebright (Rutgers U.)
Ekaterine Kortkhonjia
Vladimir Mekler
Jayanta Mukhopadhyay
Andrey Revyakin

Philip Tinnefeld (U.Heidelberg)

and all SMBs!

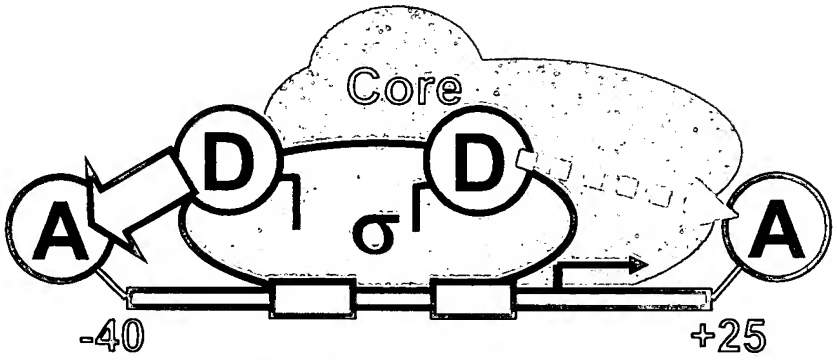


Funding: DOE, NIH

TRAILING-EDGE and LEADING-EDGE FRET:

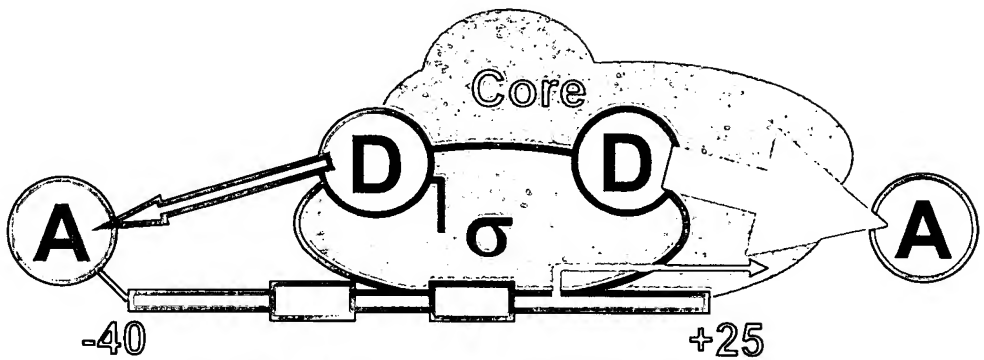
Assay of translocation of a protein relative to a nucleic acid

Trailing-edge/leading-edge FRET (TELE-FRET)



Ruler 1: High FRET

Ruler 2: Low FRET

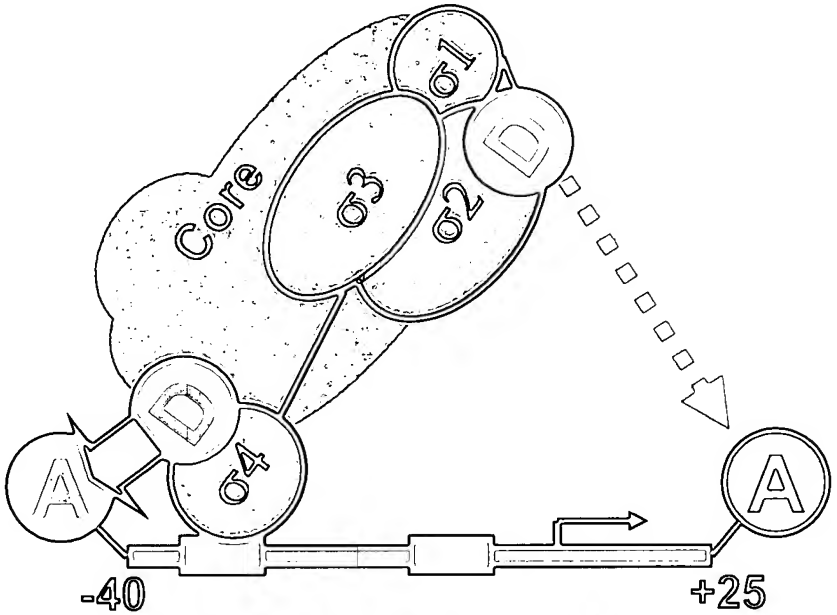


Ruler 1: Low FRET

Ruler 2: High FRET

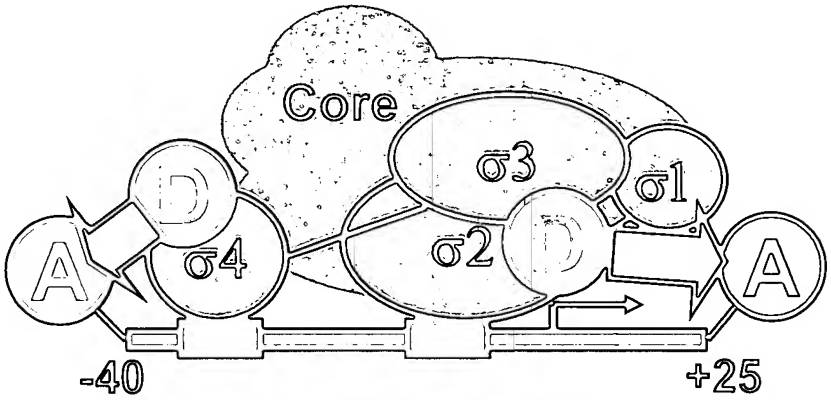
Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

Step-Sequence of formation of promoter contacts using 2 FRET rulers



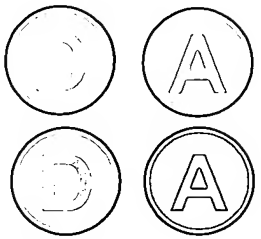
Ruler 1: High FRET

Ruler 2: Low FRET



Ruler 1: High FRET

Ruler 2: High FRET



Ruler 1

Ruler 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Shimon Weiss	Art Unit: 2877
Appl. No.: 10/561,448	Examiner: F.L. Evans
Confirmation No.: 8178	
Filed: December 20, 2005	Atty. Docket No.: 58086-226455
For: MODULATED EXCITATION FLUORESCENCE ANALYSIS	Customer No. 26694
	PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 C.F.R. § 1.131

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, being duly warned, declare the following:

1. I am a co-inventor of the subject matter described and claimed in the above-identified U.S. patent application. I have reviewed the claims of this application as currently amended.

2. I understand that the Office Action dated November 30, 2007 rejected the examined claims of this patent application under 35 U.S.C. § 102(a) over published German patent application Publication No. DE 10210737 A1 by Krieger et al. that published March 20, 2003.

3. I, together with my co-inventors, conceived the invention described and claimed in at least independent claims 1 and 21 of this application, and reduced it to practice, prior to the March 20, 2003 publication date of the cited reference. Our prior invention is evidenced by a copy of a presentation by one of the co-inventors, Achillefs Kapanidis, at the Single-Molecule Biophysics Conference in Aspen, CO on January 7, 2003, (copy attached as Exhibit A).

4. As documented by Exhibit A, my co-inventors and I conceived the invention of at least current independent claims 1 and 21, and reduced it to practice, prior to January 7, 2003.

5. The acts described above in paragraphs 3 and 4 were carried out in the United States of America, or else in a WTO member country.

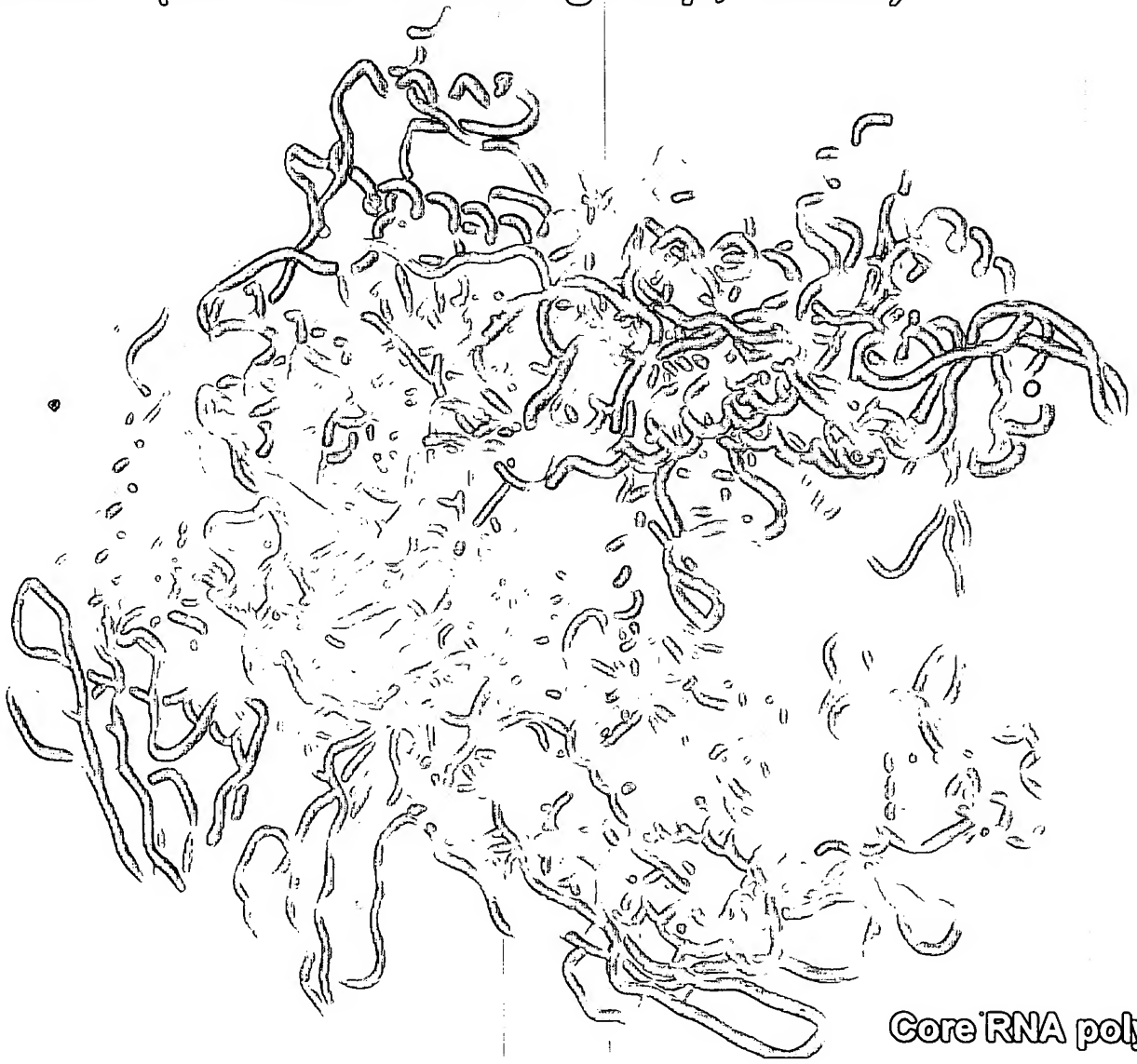
6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date	Shimon Weiss
28 May 2008 Date	Achillefs Kapanidis
Date	Ted A. Laurence
Date	Nam K. Lee

Exhibit A

Molecular Machines at Work:
Single-Molecule Analysis of Transcription by RNA Polymerase

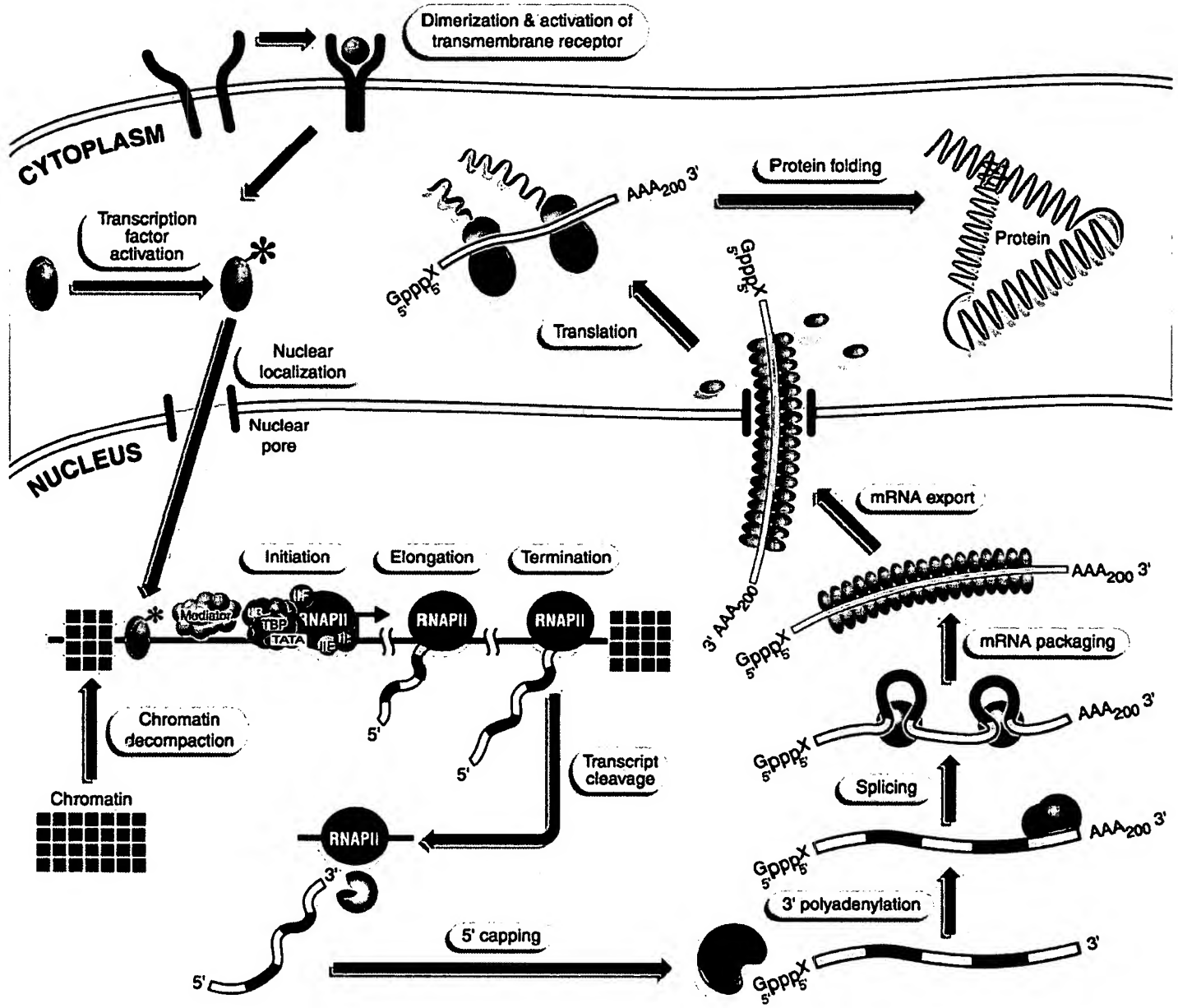
Achillefs Kapanidis (Shimon Weiss' group, UCLA)



Core RNA polymerase (Darst lab)

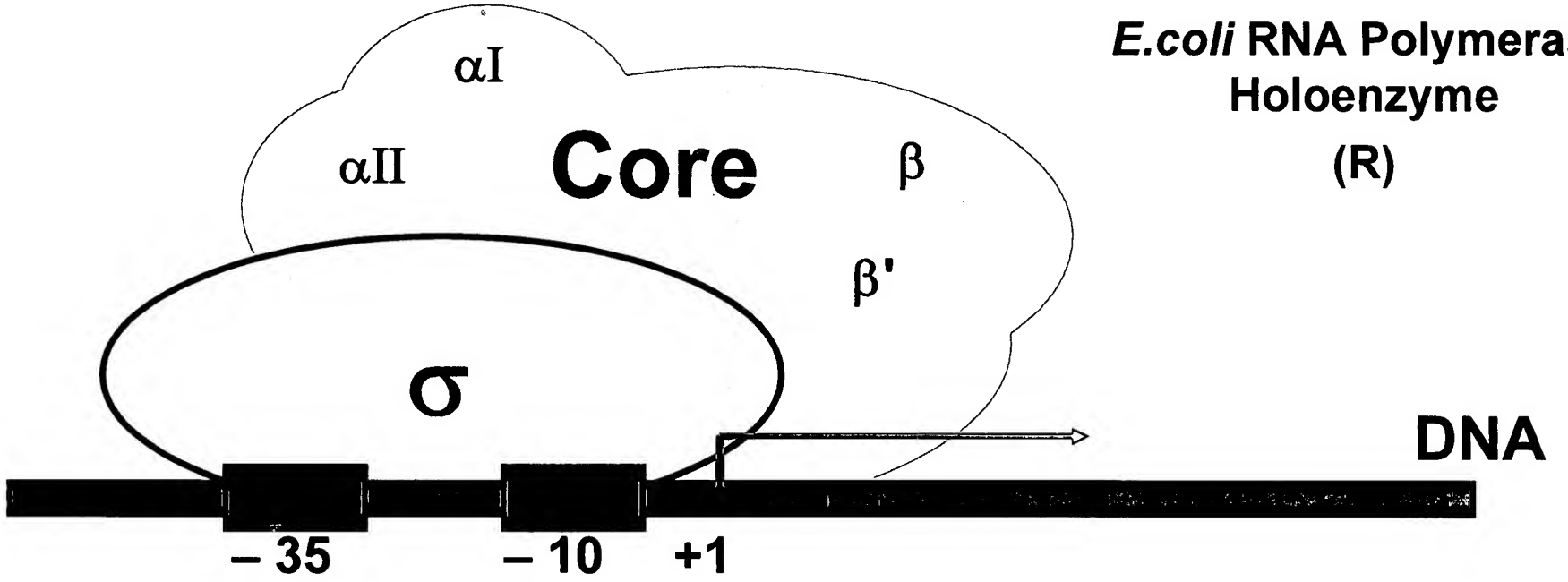
Single-Molecule Biophysics Conference: Aspen, Jan.7, 2003

GENE EXPRESSION:
The path from gene to protein

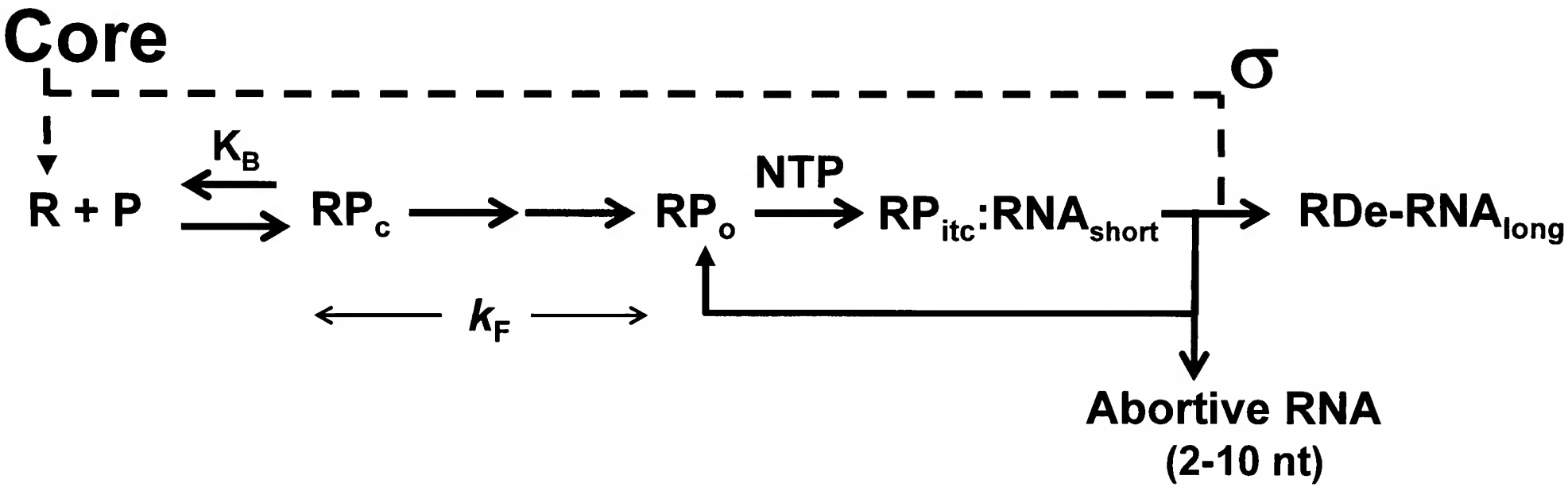


Orphanides and Reinberg, 2002

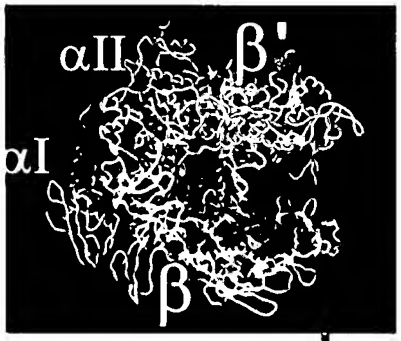
TRANSCRIPTION INITIATION



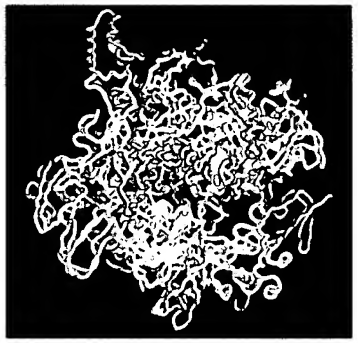
E.coli RNA Polymerase Holoenzyme (R)

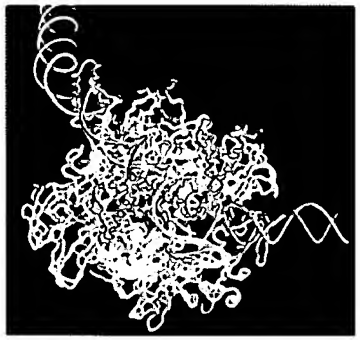


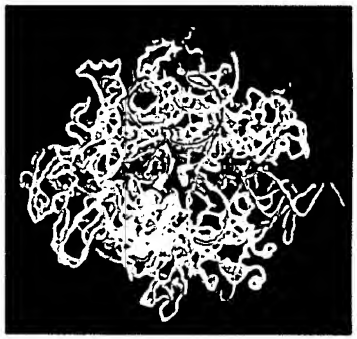
STRUCTURAL ASPECTS OF TRANSCRIPTION



RNAP core ($\alpha_2\beta\beta'$)


RNAP holoenzyme (core + σ)


RP_o


RD_e (model)

X-ray structures → static snapshots of the machine

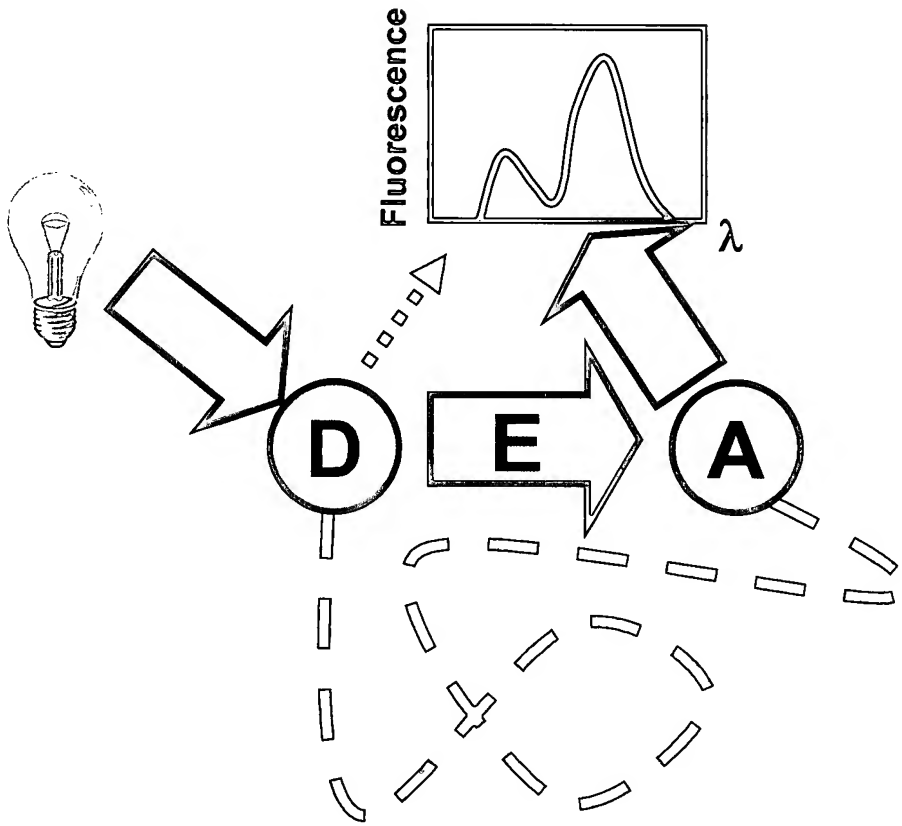
SMD: "movie" of the dynamic process

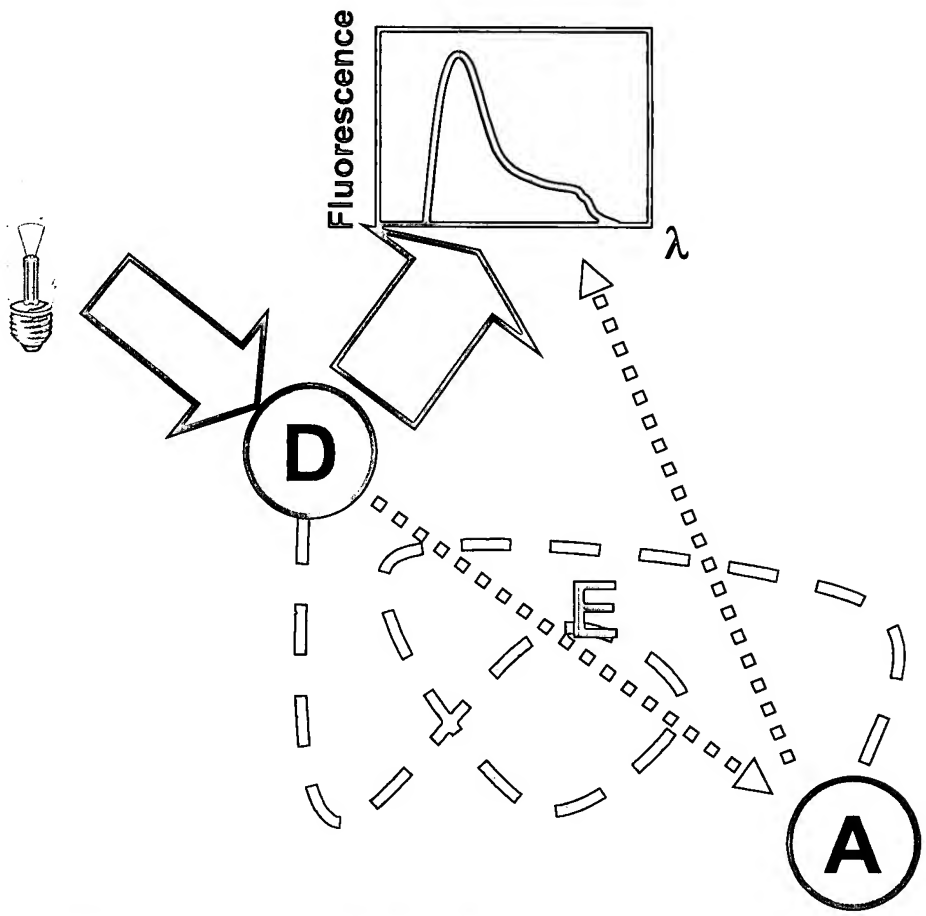
Structure
Dynamics
Local Environment → **Intermediates** **Kinetics** → **Timing** **of Events** → MECHANISM

Young *et al.*, 2002

FÖRSTER RESONANCE ENERGY TRANSFER (FRET):

A "MOLECULAR RULER" FOR THE 2-10 nm REGIME





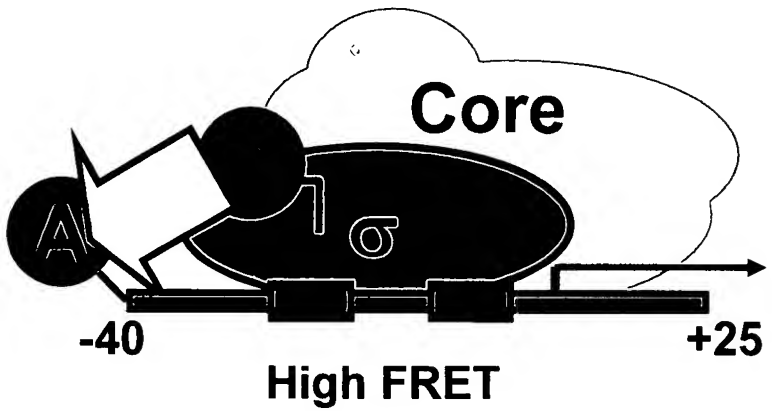
$$\text{FRET Efficiency, } E = [1 + (R/R_o)^6]^{-1}$$

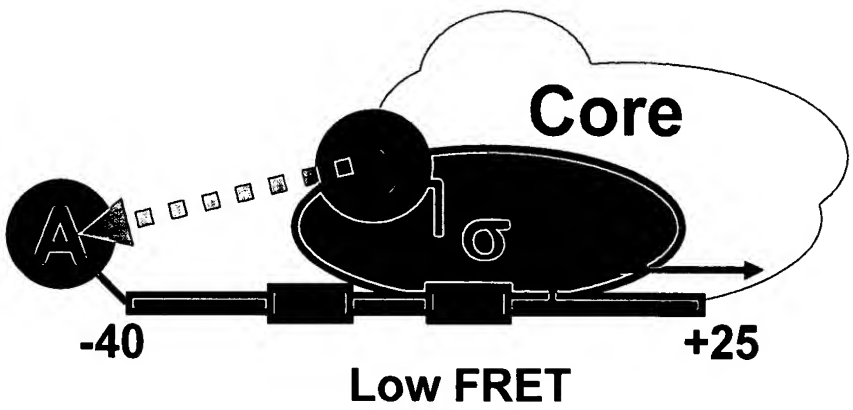
R = D-A Distance

TRAILING-EDGE and LEADING-EDGE FRET:

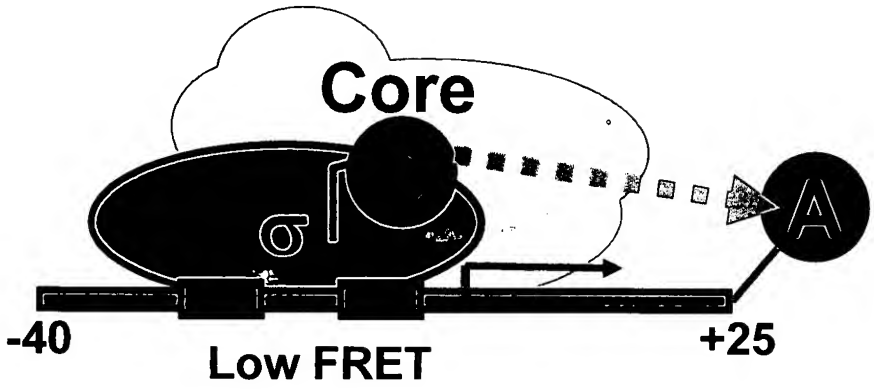
Assay of translocation of a protein relative to a nucleic acid

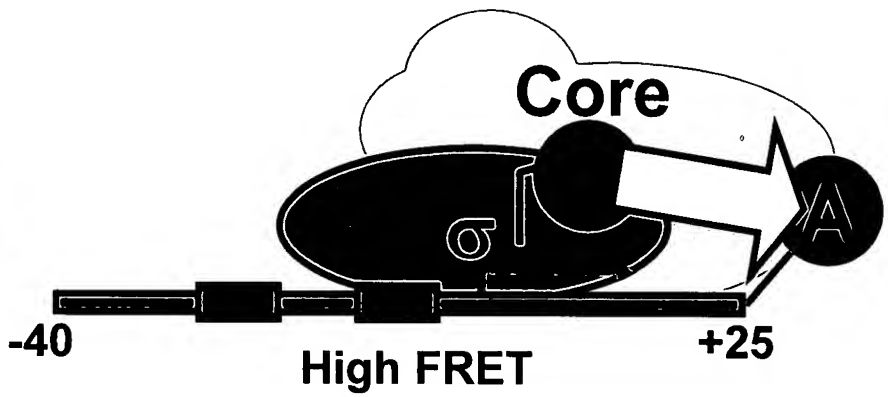
Trailing-edge FRET





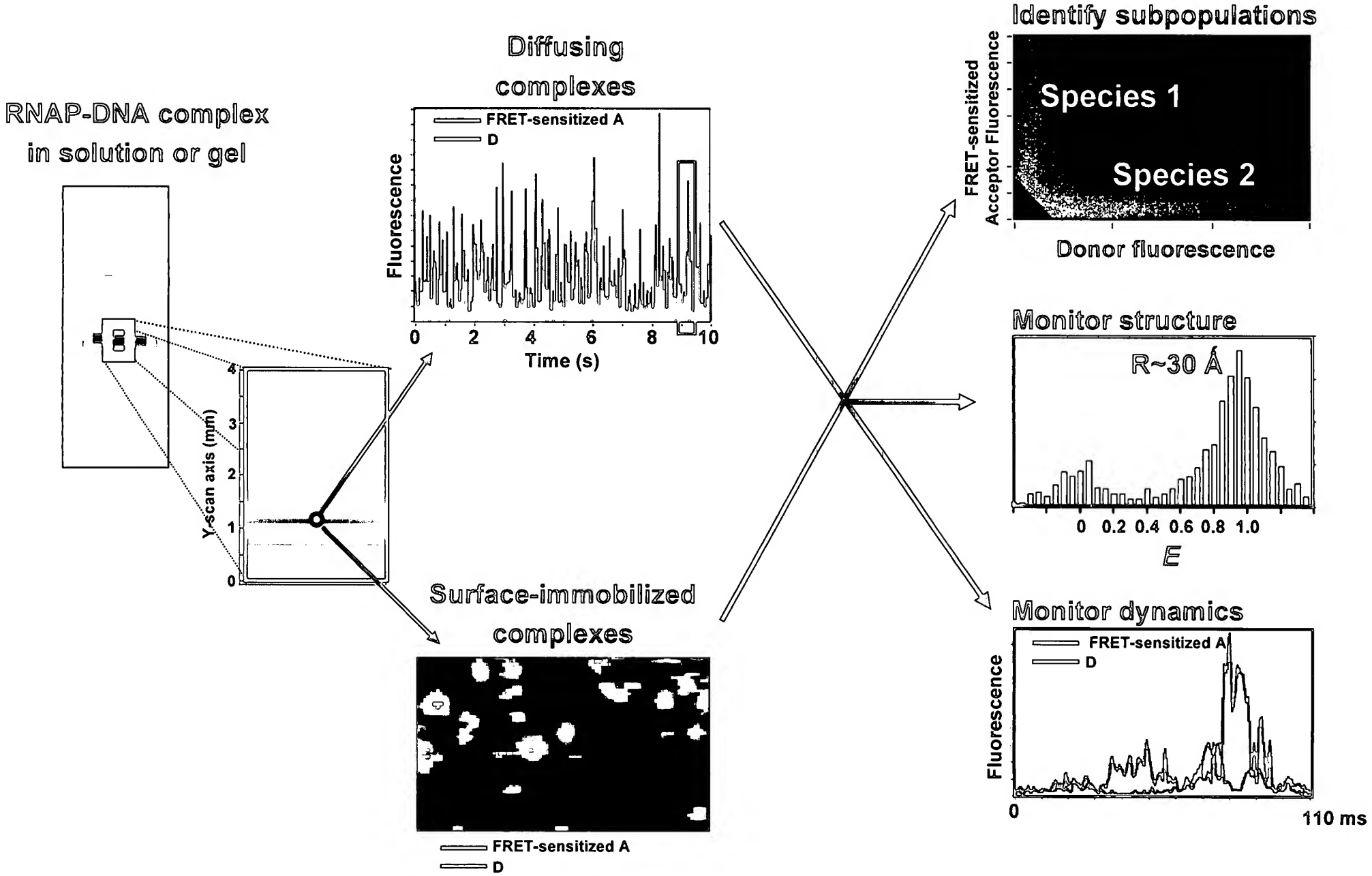
Leading-edge FRET





Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

sp-FRET ON RNAP-DNA COMPLEXES

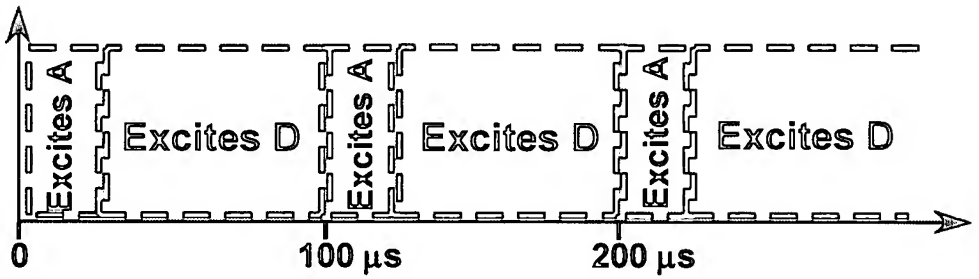


LIMITATIONS OF SINGLE-LASER EXCITATION spFRET

- Complex FRET Acceptor photophysics
  - "Dark" states→D-only peak
  - Photobleaching→ D-only peak
  - Intermittency ("Blinking")
- Complex FRET Donor photophysics
  - Intermittency
  - Transient QY changes
- Limited discrimination ability in the FRET coordinate
  - FRET range of 0-0.3 not easily accessible
- Variable fluorescence contamination
  - Adds variable counts to D-only peak

sp-FRET USING ALTERNATE LASER EXCITATION (ALEX)

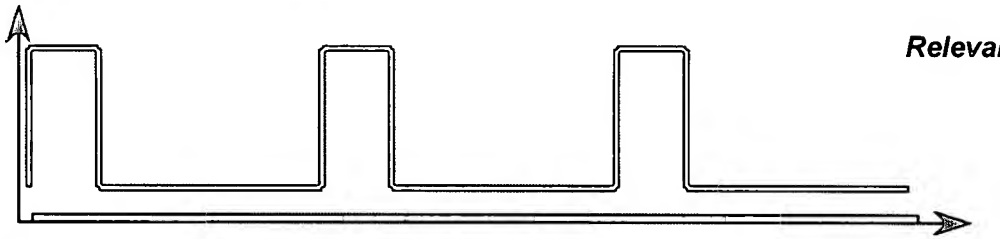
EXCITATION PROFILE



EMISSION PROFILES

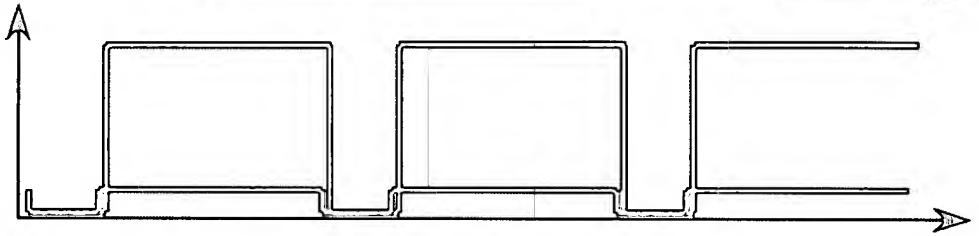
Relevant transcription species

A-only species



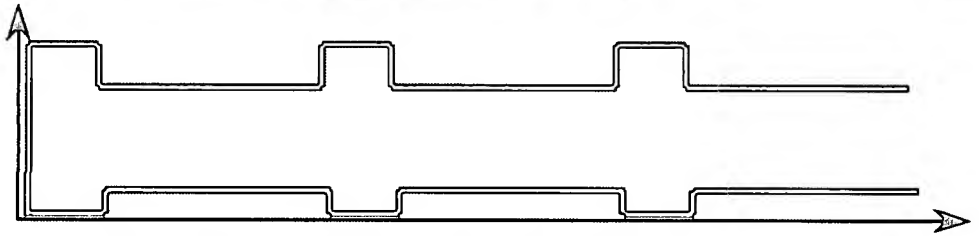
DNA^{A}

D-only species



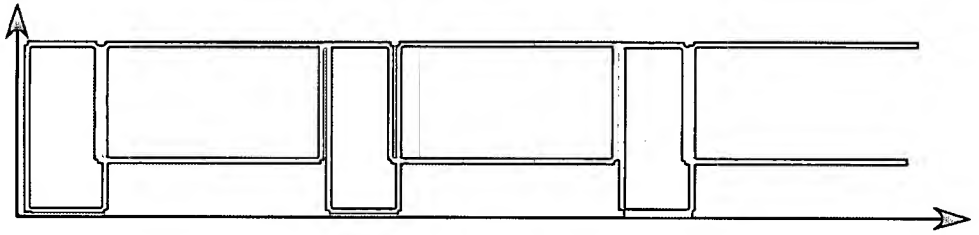
$RNAP\sigma^{D}$

D-A species, high FRET



RPo^{D-A}, Trailing-edge

D-A species low FRET



RPo^{D-A}, Leading-edge

EQUATIONS

Energy transfer ratio (*E*)

$$E = \frac{F^{DA}_{670em,514ex}}{F^{DA}_{670em,514ex} + F^{DA}_{580em,514ex}}$$

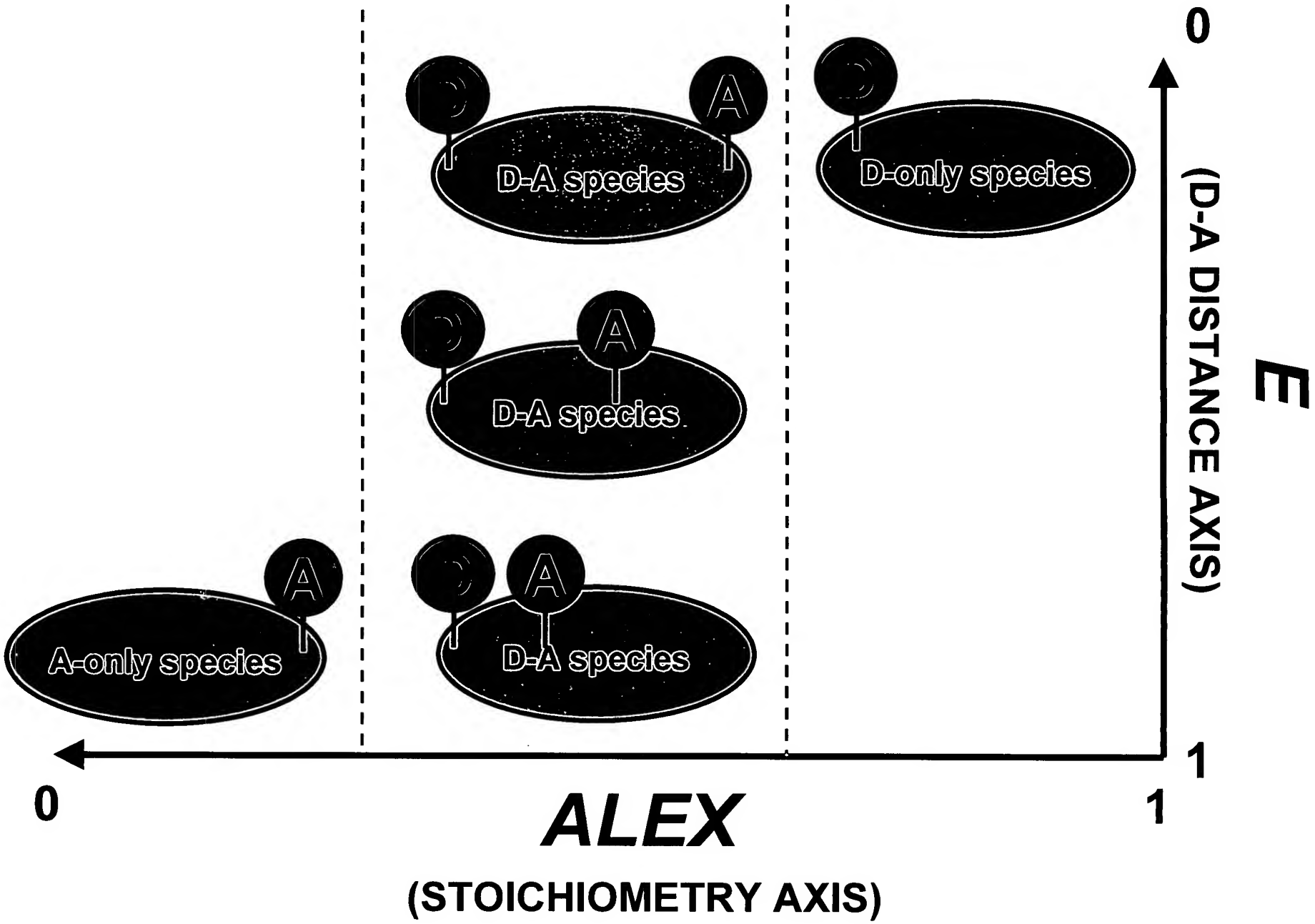
ALEX-based ratio (*ALEX*)

$$ALEX = \frac{F_{514ex}}{F_{514ex} + F_{638ex}} = \frac{F_{670em,514ex} + F_{580em,514ex}}{F_{670em,514ex} + F_{580em,514ex} + F_{670em,633ex}}$$

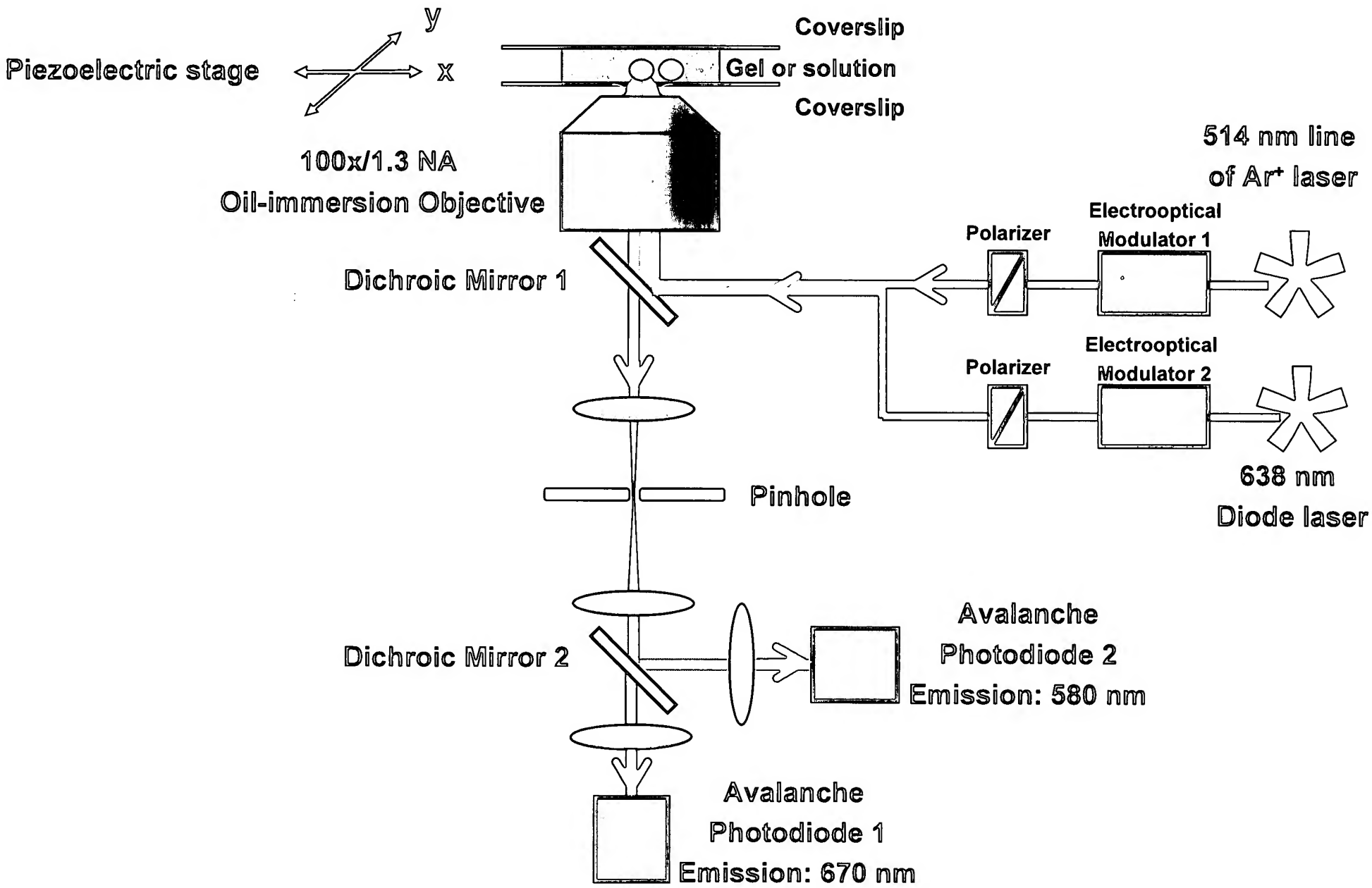
$$ALEX = \frac{0+100}{0+100+0} \sim 1.0$$

$$ALEX = \frac{50+50}{50+50+100} \sim 0.5$$

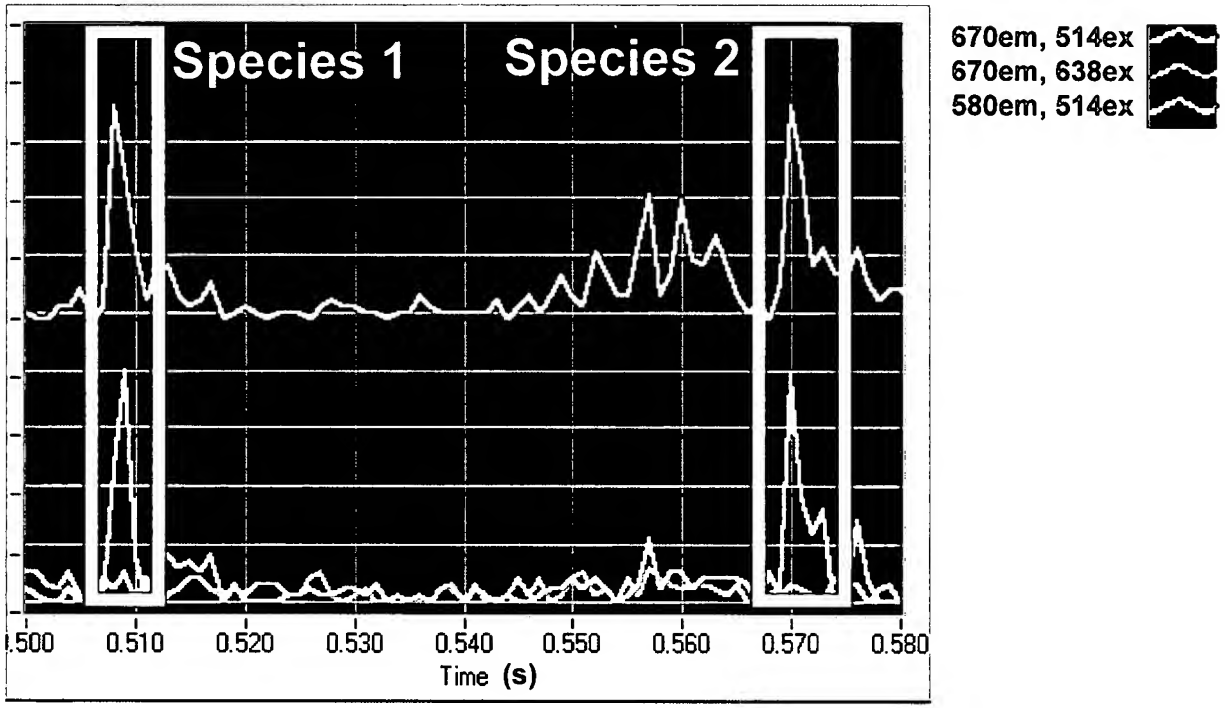
$$ALEX = \frac{0+0}{0+0+100} \sim 0.0$$


SORTING SPECIES USING E, ALEX
D-A species
D-only species
D-A species
A-only species
D-A species
0
(D-A DISTANCE AXIS)
E
1
0
ALEX
1
(STOICHIOMETRY AXIS)

ALEX SINGLE-MOLECULE CONFOCAL MICROSCOPY

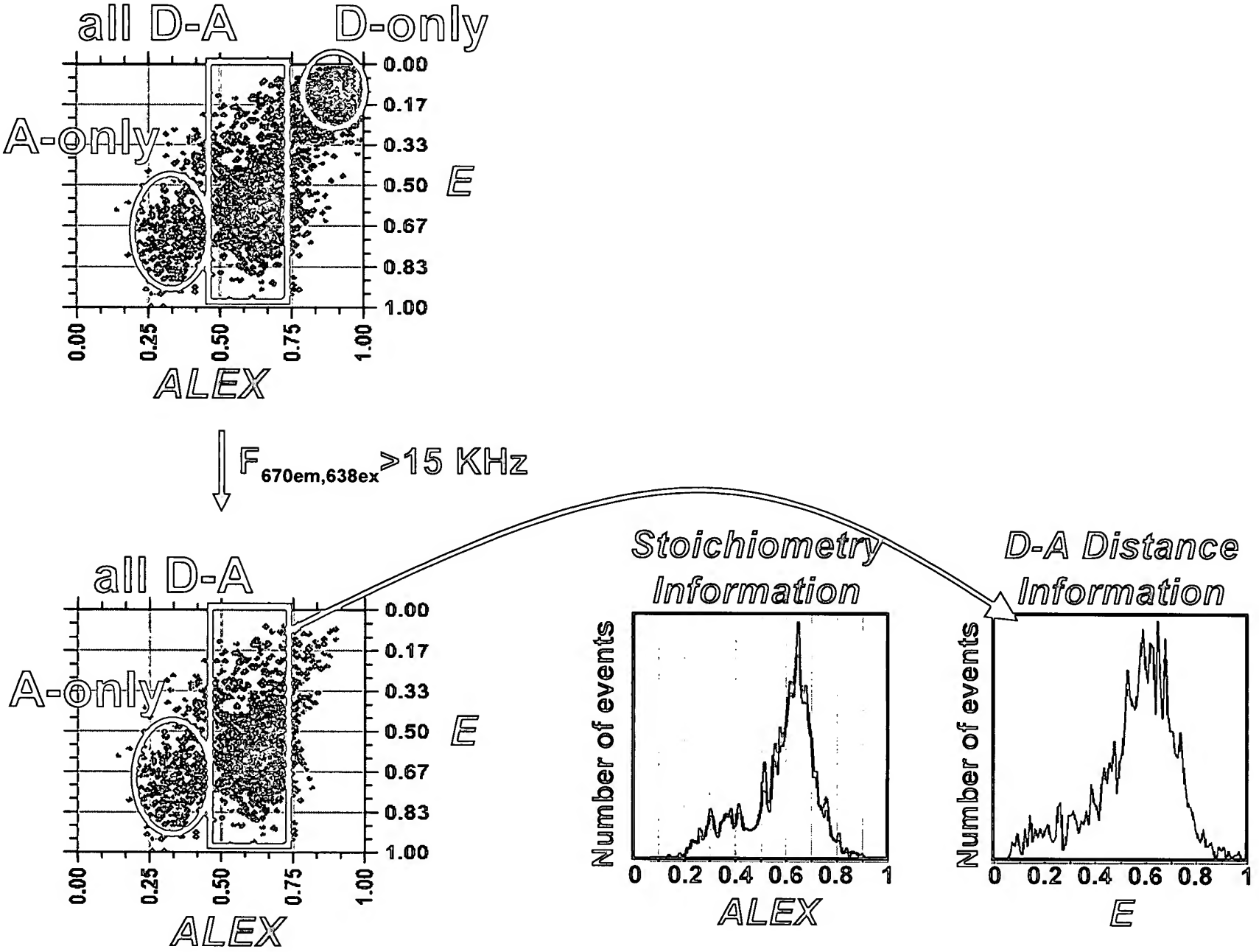


DATA ANALYSIS FOR INDIVIDUAL SPECIES

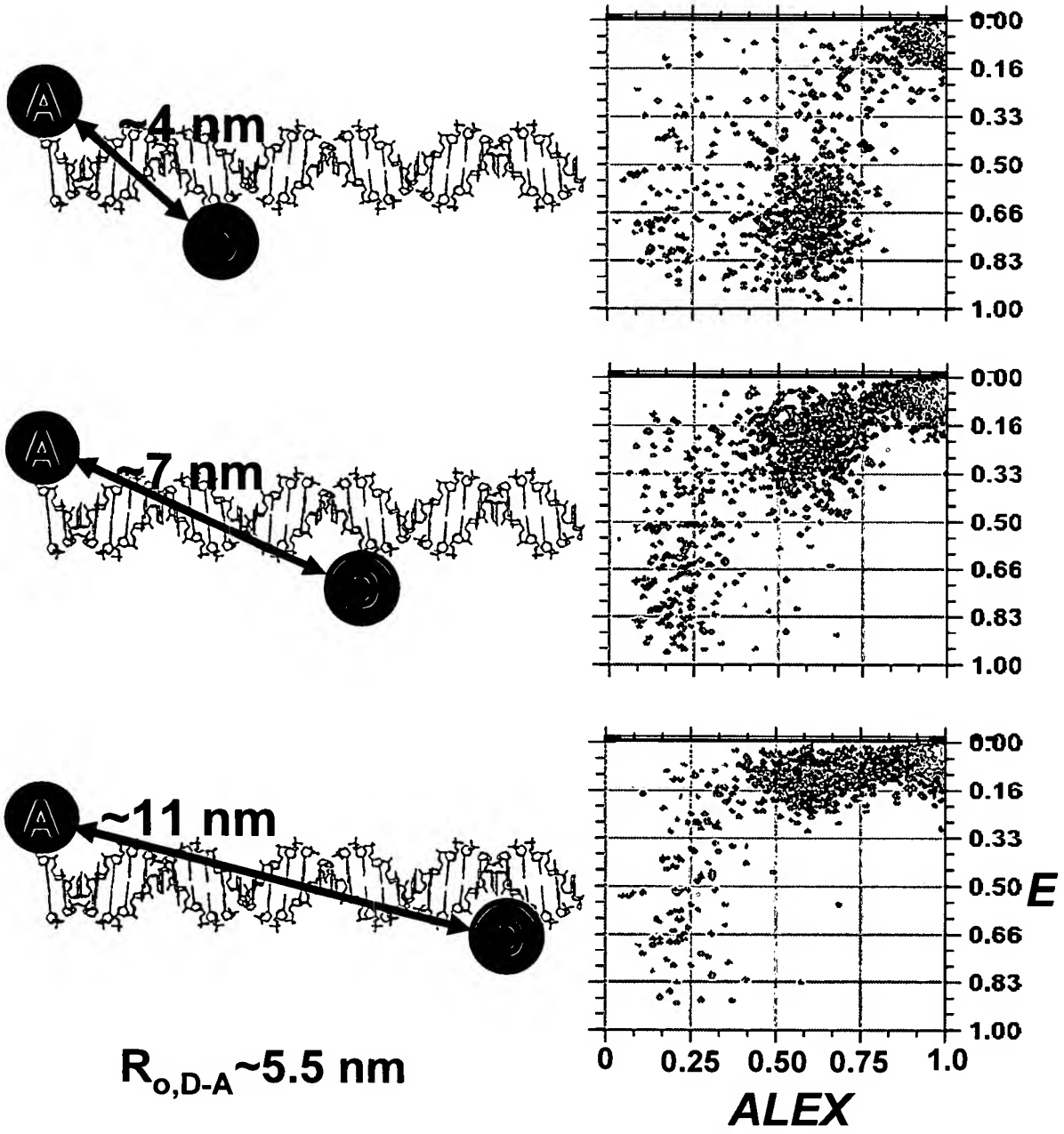


	Species 1	Species 2
670em, 514ex	71	85
670em, 638ex	69	93
580em, 514ex	7	11
FRET-sensitized A	52	60
E, simplified	91%	88%
E, FRET-sensitized A	91%	77%
ALEX	0.49	0.66

DATA ANALYSIS USING *E-ALEX* 2-D HISTOGRAMS

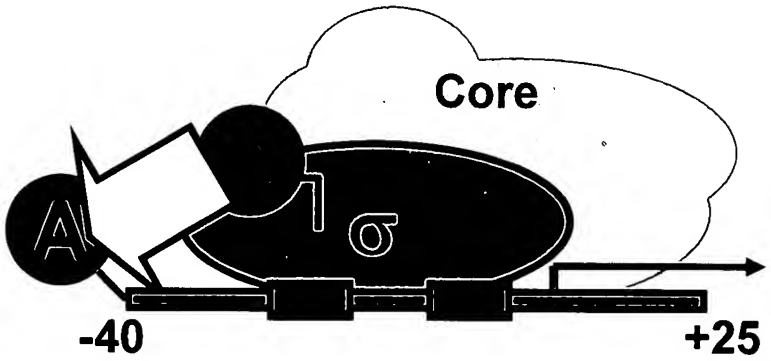


MODEL SYSTEMS: dsDNA



USING TRAILING-EDGE sp-FRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE

OPEN COMPLEX

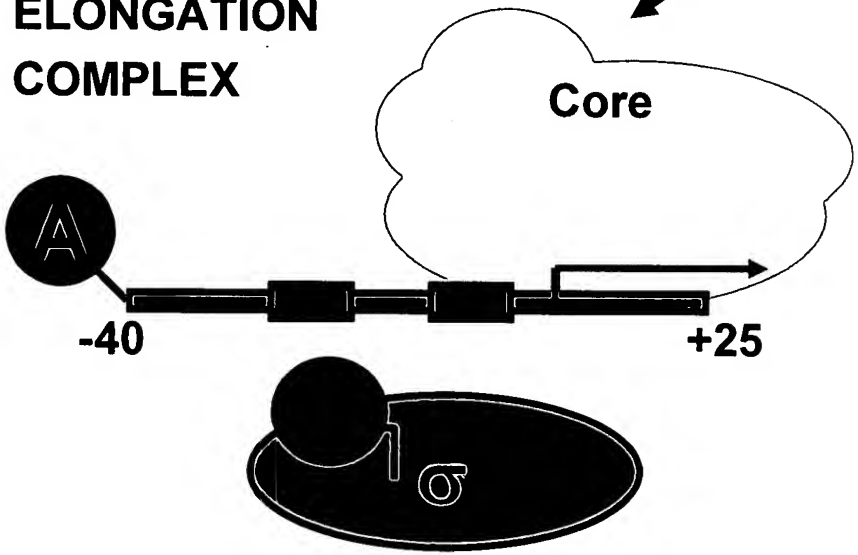


D and A co-localize; High E

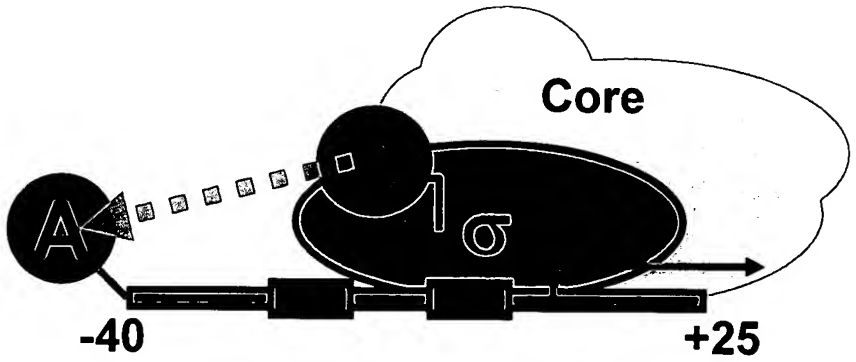
σ release model

σ non-release model

ELONGATION COMPLEX



D and A do *not* co-localize; Zero E



D and A co-localize; Zero or low E

Mukhopadhyay *et al.*, 2001

TRAILING-EDGE spFRET

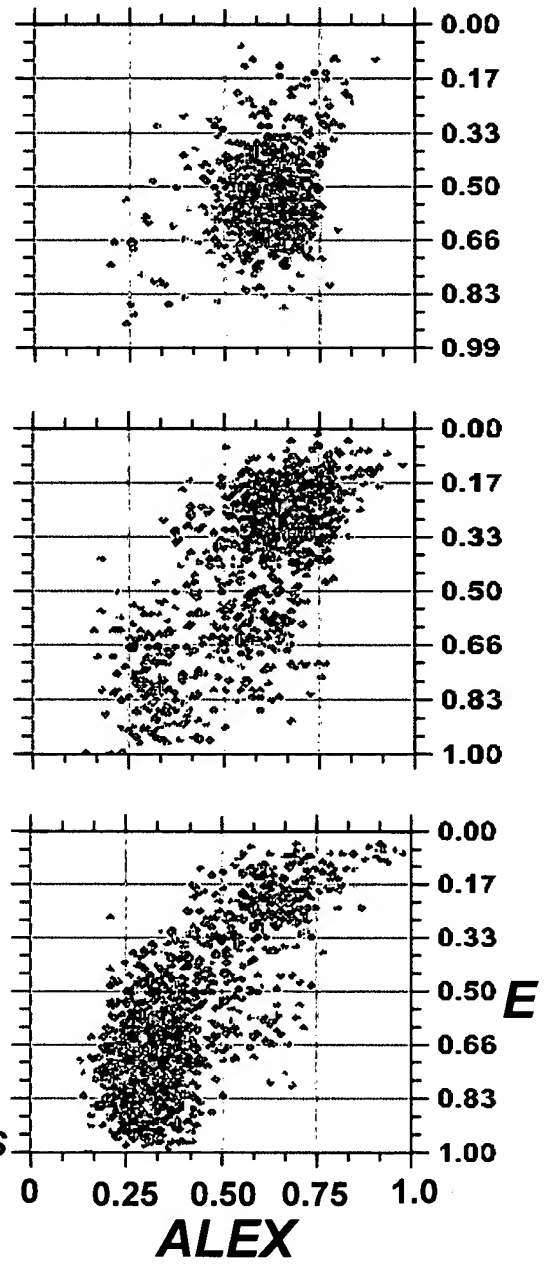
RNAP$\sigma^{TMR,569}$→lacUV5-11$^{Cy5,-40}$

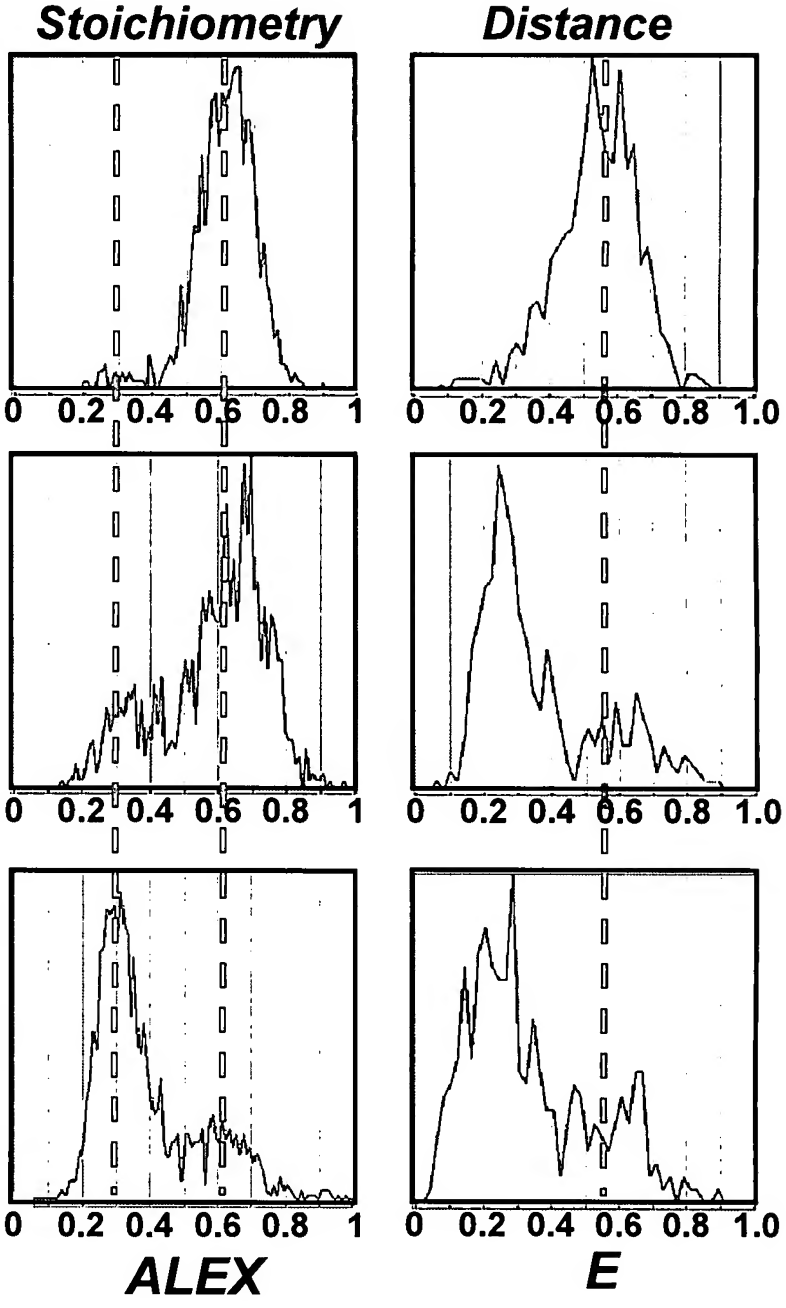
RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

RPo + ApA
+ 60 μM NTPs
(chase)

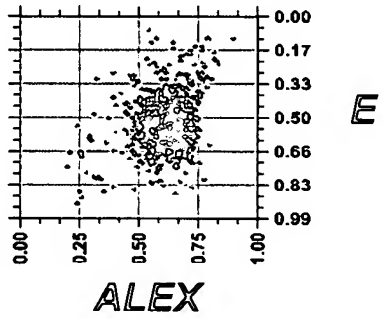
ABILITY OF STALLED COMPLEXES TO RESUME TRANSCRIPTION

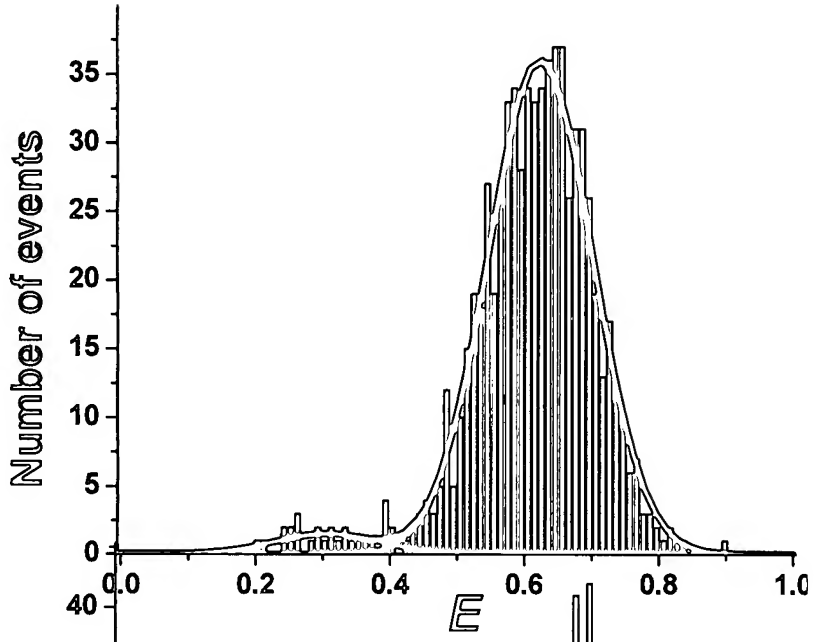




DIRECT OBSERVATION OF SIGMA NON-RELEASE: TRAILING-EDGE spFRET

$RP_{itc,2}$





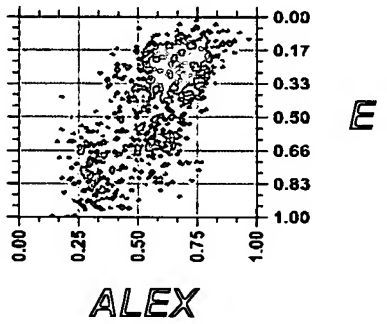
~5% dissociation

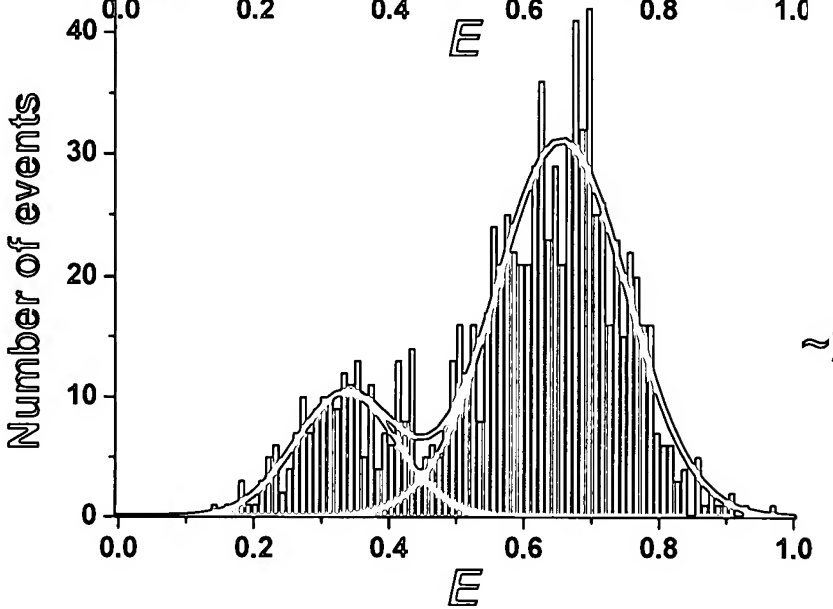
% Dissociation =

(A-only)

(A-only)+(all D-A)

$RD_{e,11}$

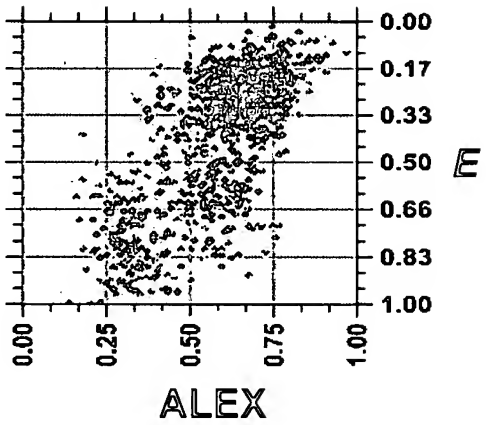


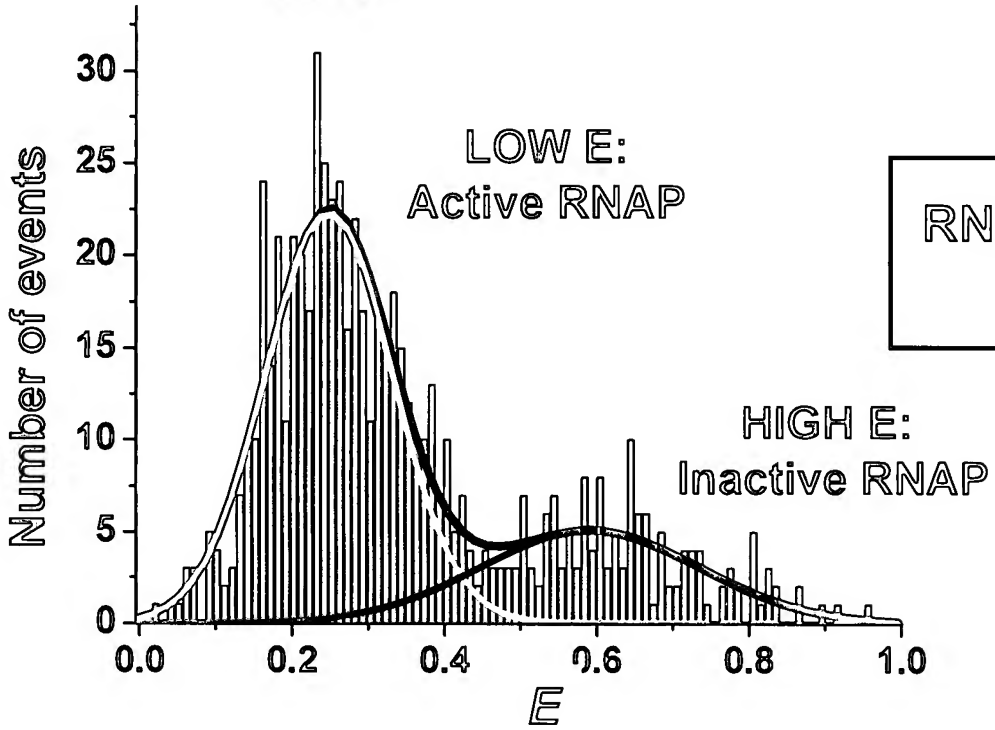


~20% dissociation

E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: TRAILING-EDGE spFRET

RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

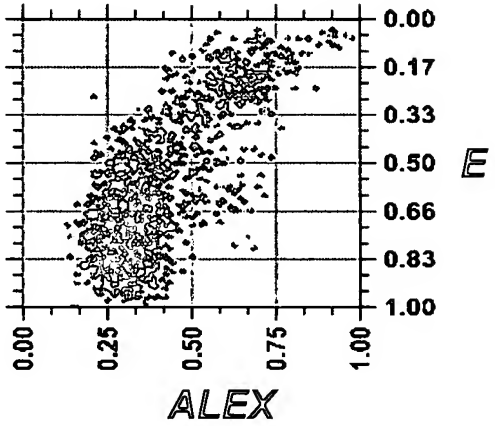


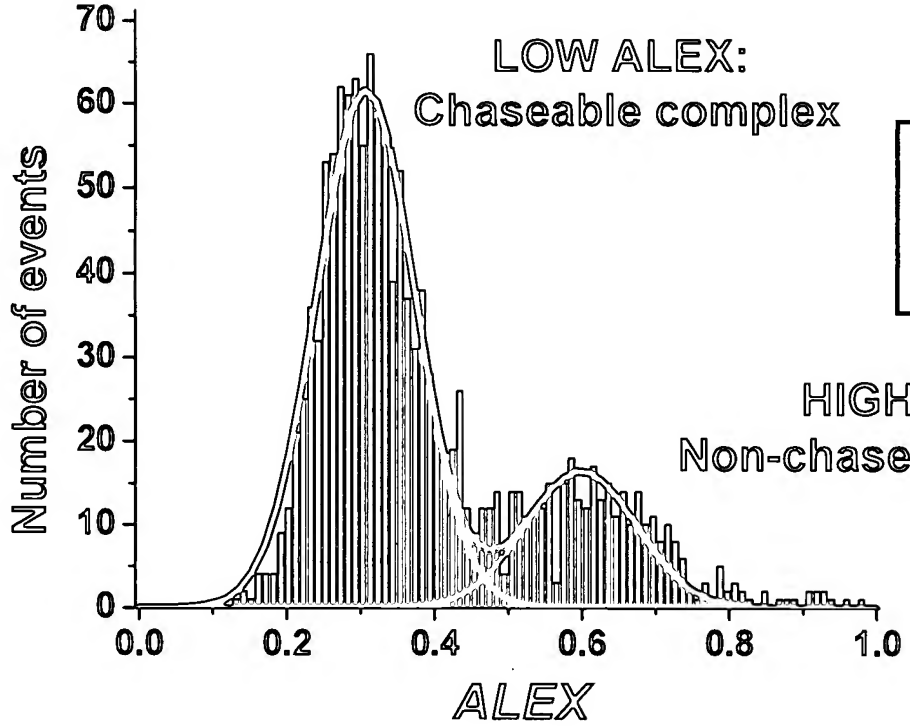


RNAP translocational activity ~ 70%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": TRAILING-EDGE spFRET

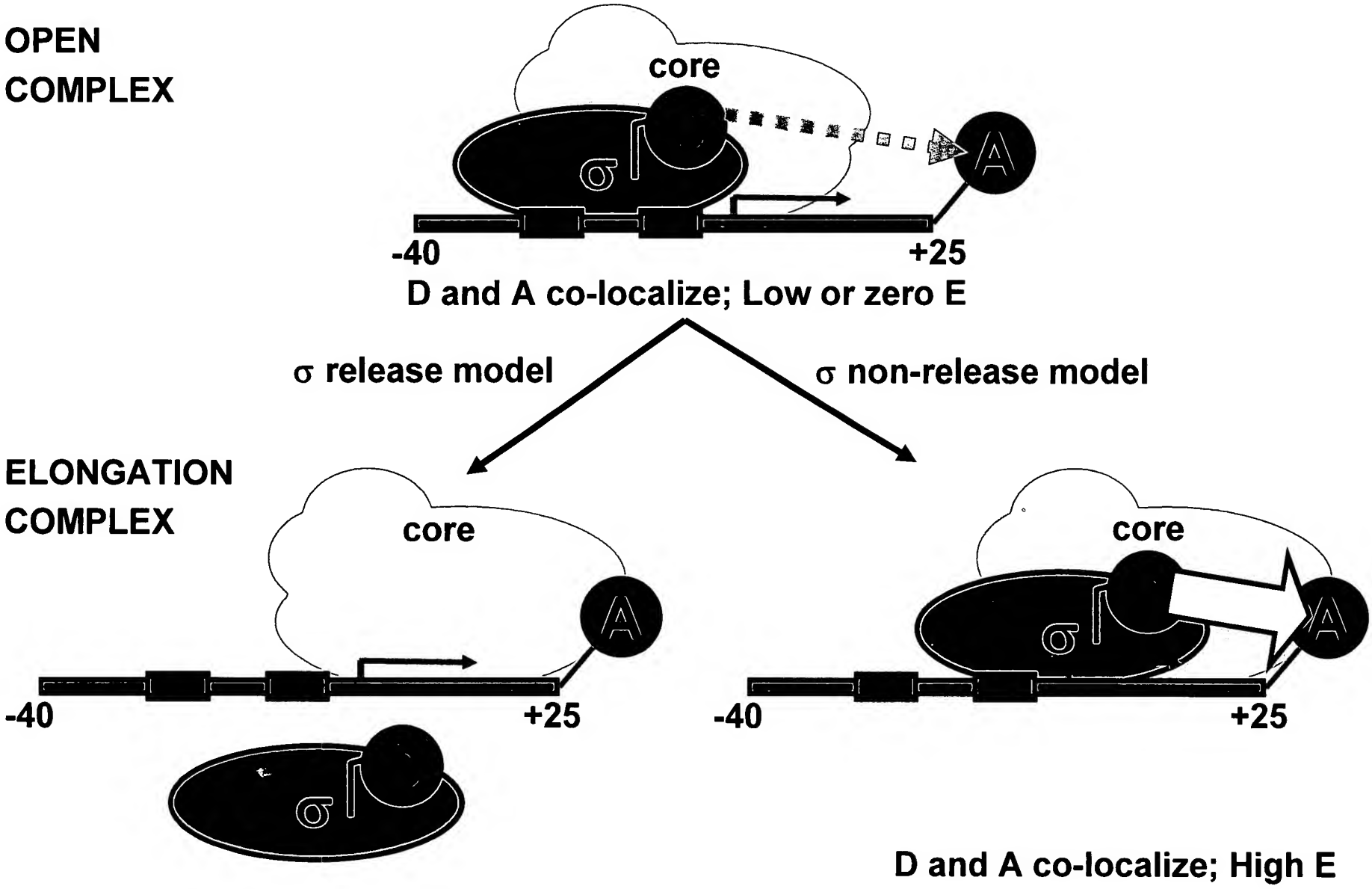
RPo + ApA + 60 μM NTPs (chase)





RNAP "chaseability"
~ 80%

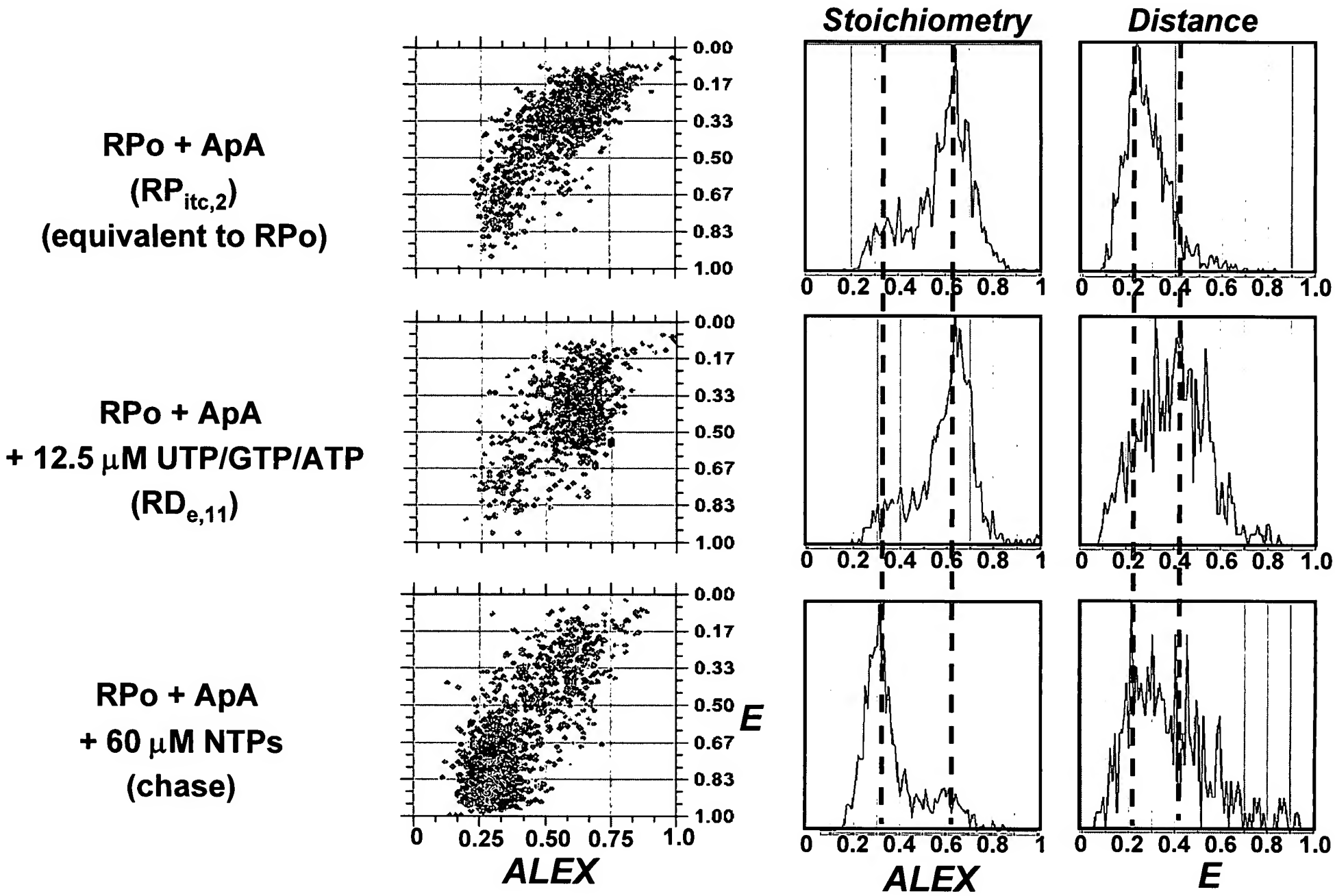
USING LEADING-EDGE spFRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE



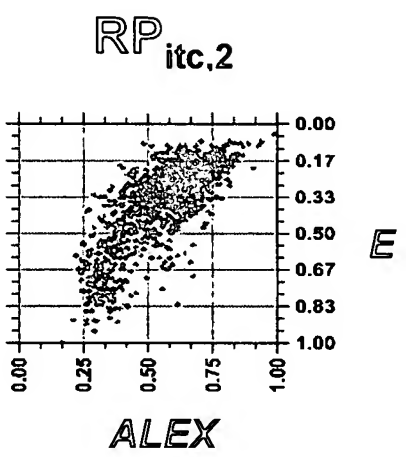
Mukhopadhyay *et al.*, 2001

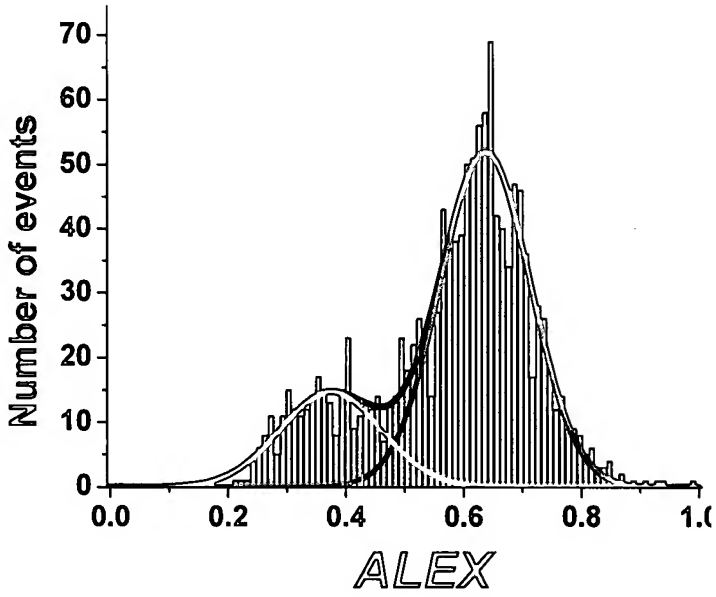
LEADING-EDGE spFRET

$RNAP\sigma^{TMR,366} \rightarrow lacUV5\text{-}11^{Cy5,+25}$

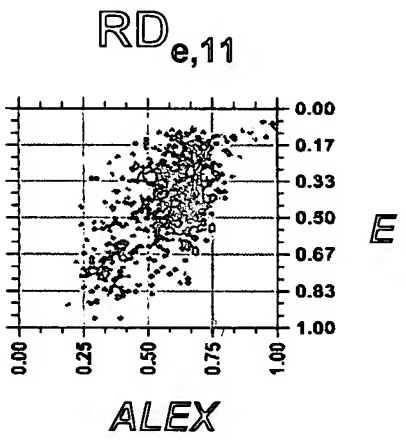


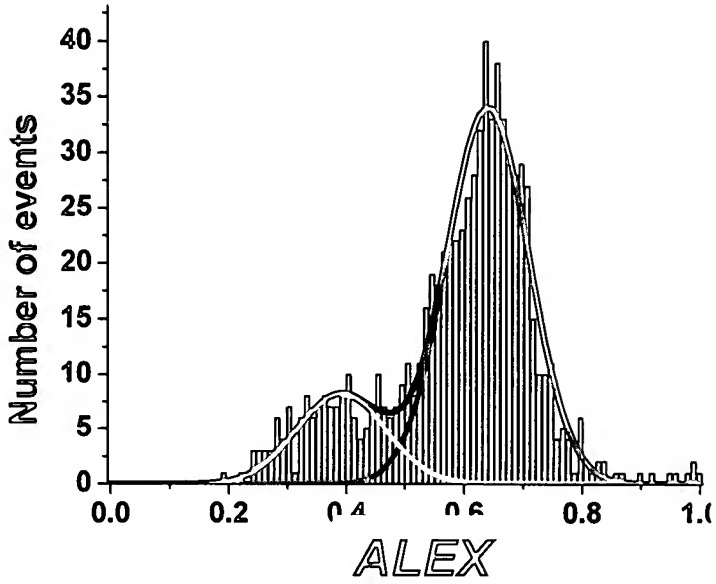
DIRECT OBSERVATION OF SIGMA NON-RELEASE: LEADING-EDGE spFRET





~20% dissociation

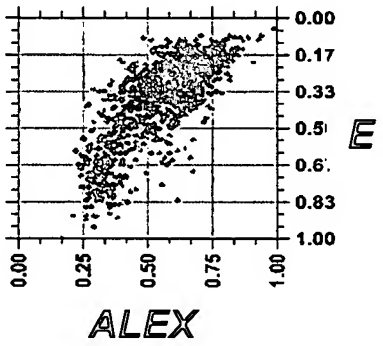


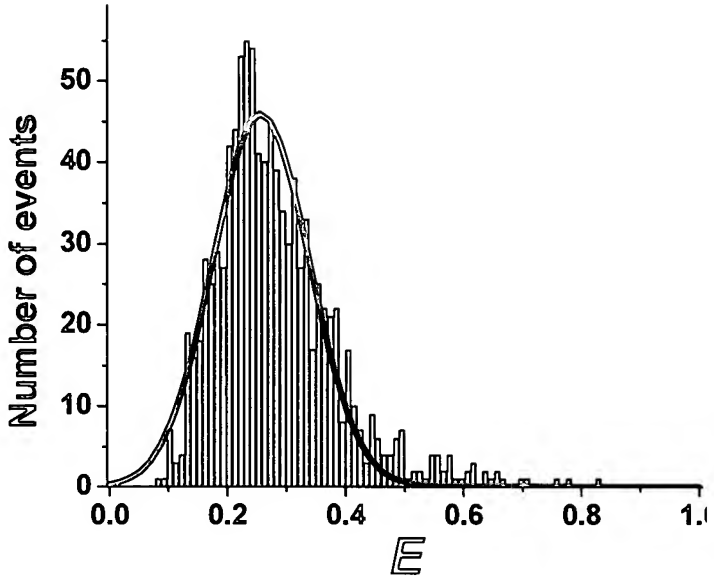


~20% dissociation

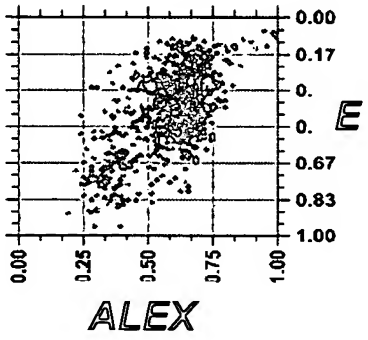
E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: LEADING-EDGE spFRET

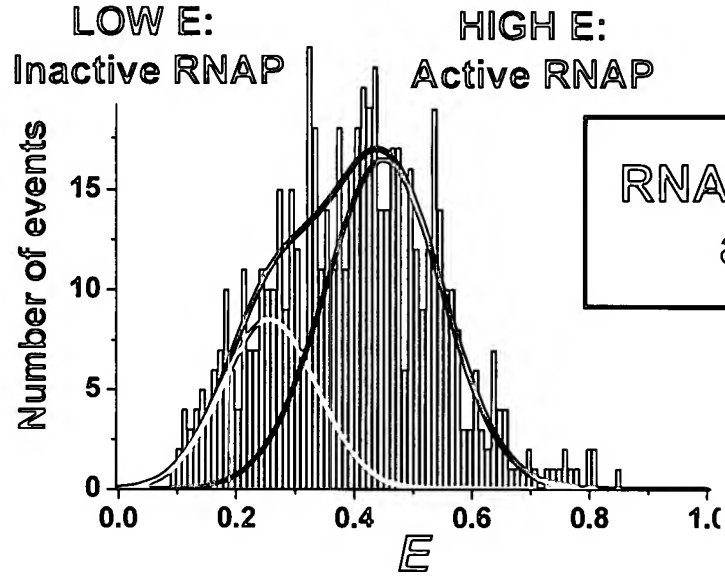
RPo + ApA ($RP_{itc,2}$)





RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

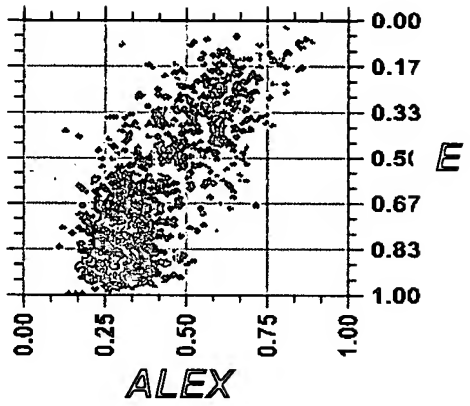


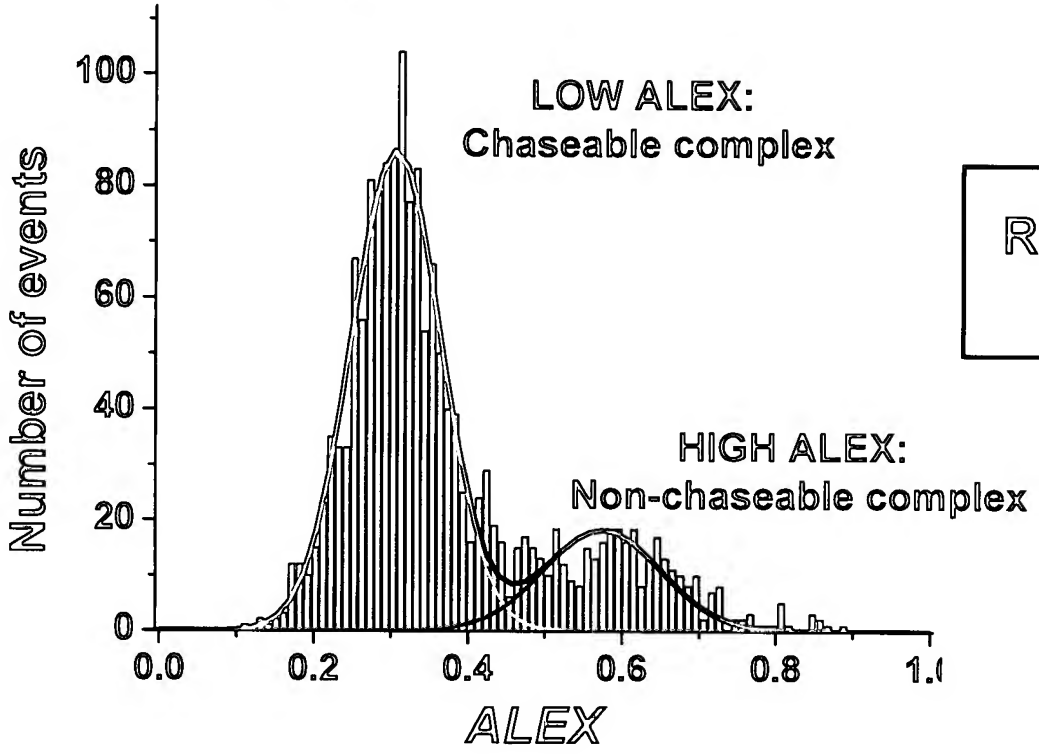


RNAP translocational activity = 72%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": LEADING-EDGE spFRET

RPo + ApA + 60 μM NTPs (chase)

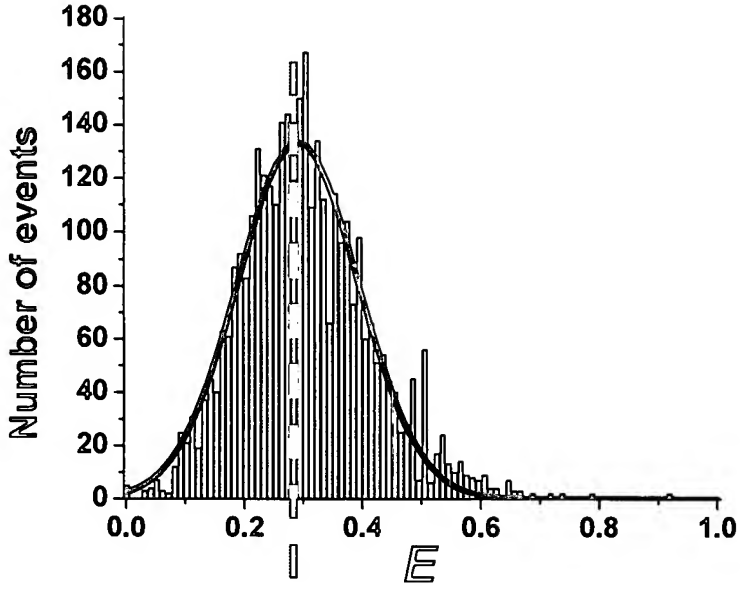




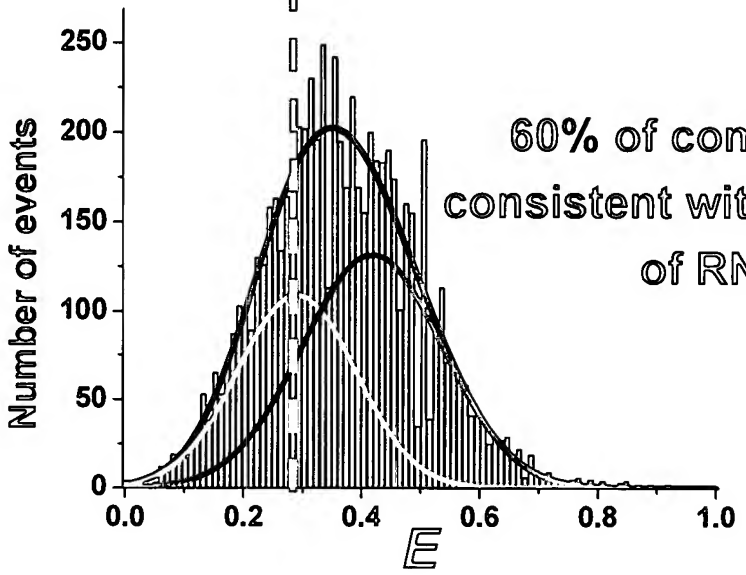
RNAP "chaseability" = 80%

LEADING-EDGE spFRET DETECTS MOVEMENT OF LEADING EDGE DURING ABORTIVE INITIATION

RPo + ApA
($RP_{itc,2}$)



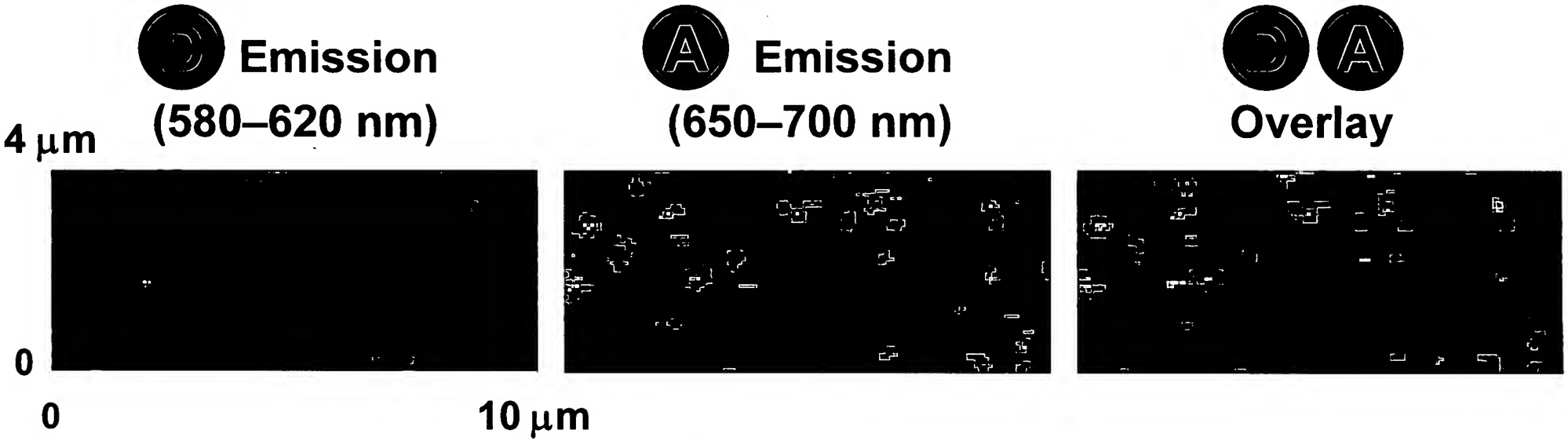
RPo + ApA
+ 25 μM UTP/GTP
($RD_{e,7}$)



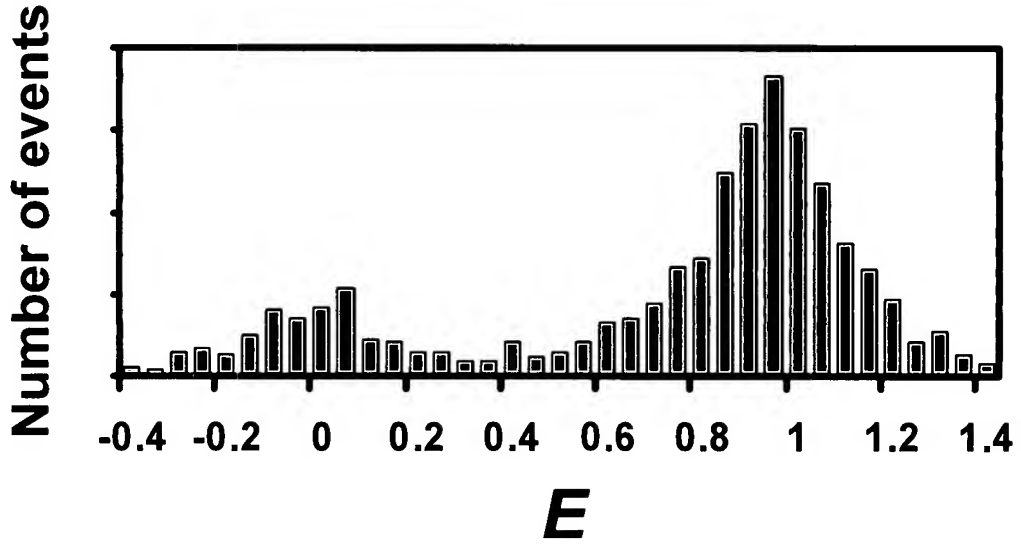
60% of complexes show higher *E*; consistent with downstream movement of RNAP leading edge

TRAILING-EDGE spFRET ON SURFACE-IMMOBILIZED RP_o COMPLEXES

Excitation: 514 nm line of Ar^+ laser

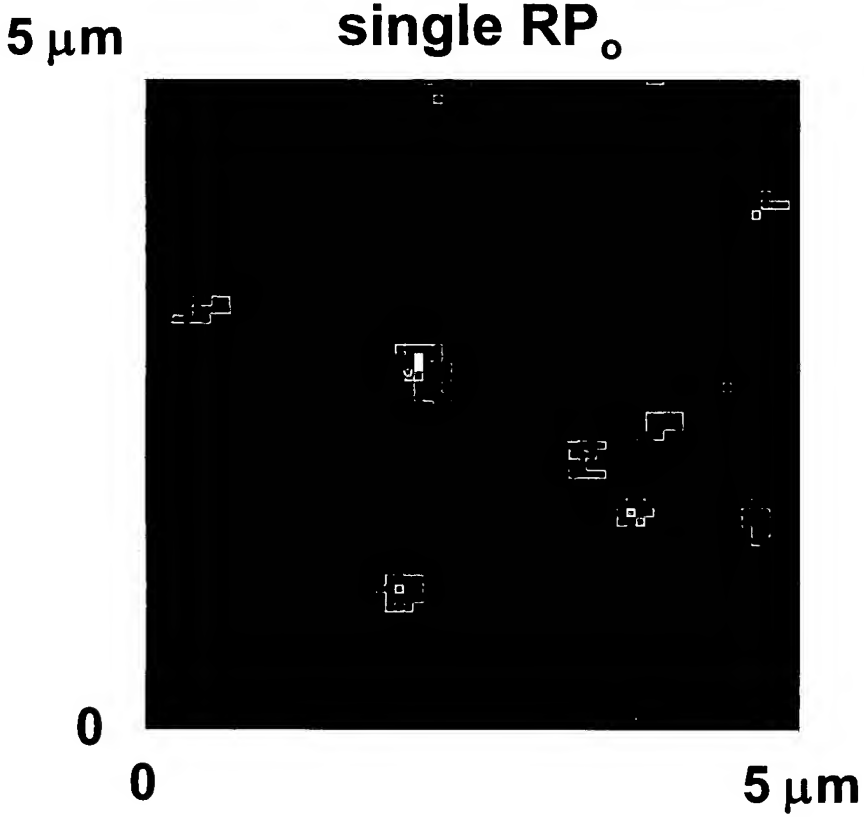


$$E = \frac{I_A}{I_A + \gamma I_D}$$

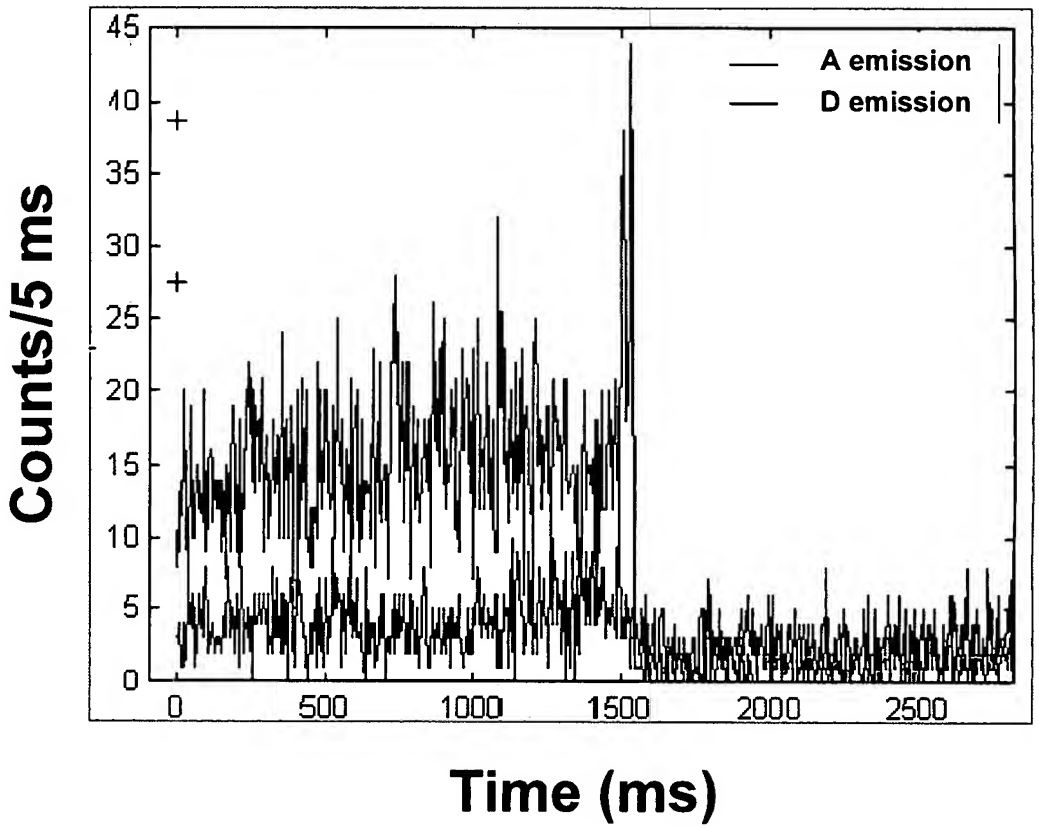


IMAGING AND TIME-TRAJECTORIES OF SINGLE RP_o COMPLEXES

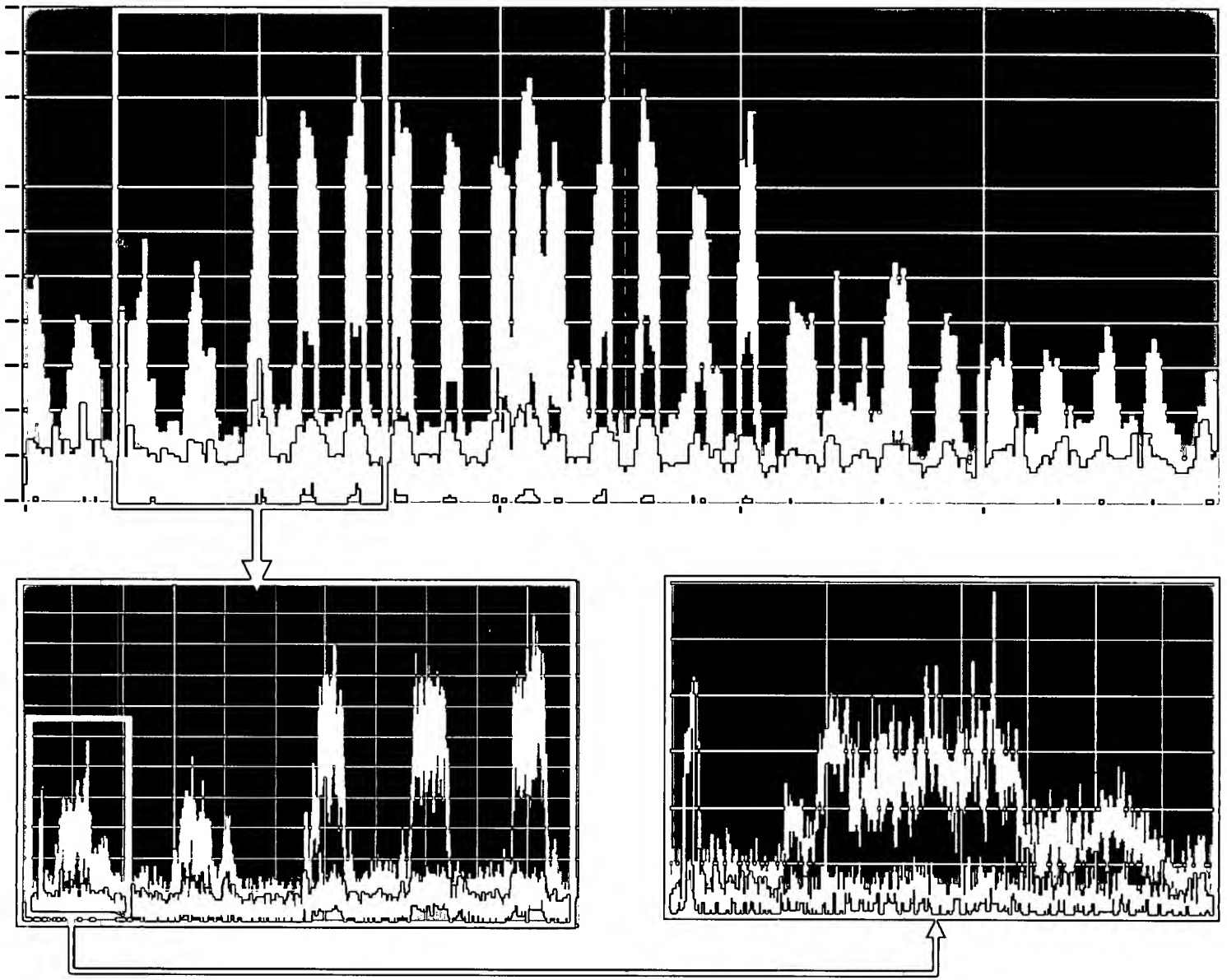
Single-step photobleaching: evidence for imaging single RP_o



Time-trajectory for a single RP_o showing TE-FRET



MONITORING SINGLE-ENZYME DYNAMICS ON IMMOBILIZED MOLECULES



CONCLUSIONS

- Developed robust assays for analysis of structure, dynamics, and activity of protein-DNA complexes
- Confirmed sigma presence in early elongation complexes
- Determined activity for translocation and for chase reactions
- Detected movement of leading edge during abortive initiation
- Future work:
    - Abortive initiation mechanism
    - Sigma dynamics at various transcription steps

ACKNOWLEDGEMENTS

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Xavier Michalet

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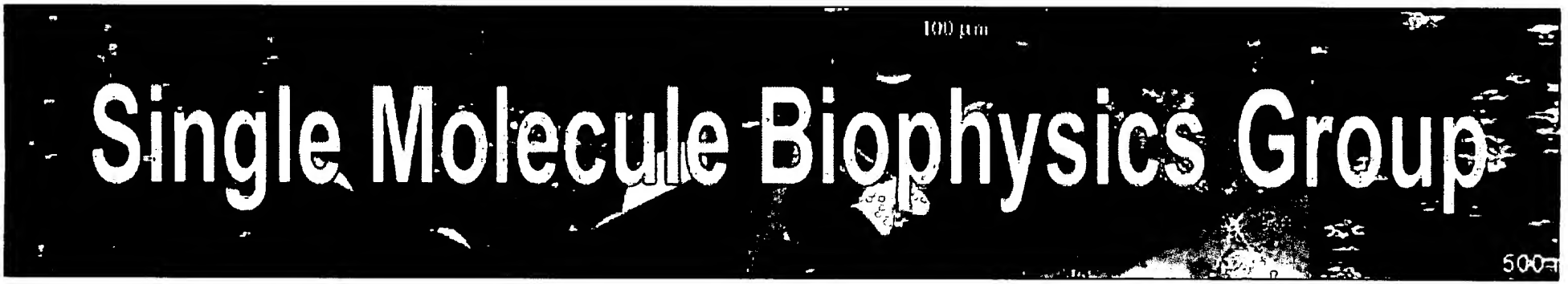
Ekaterine Kortkhonjia
Vladimir Mekler
Jayanta Mukhopadhyay
Andrey Revyakin

Philip Tinnefeld (U.Heidelberg)

and all SMBs!

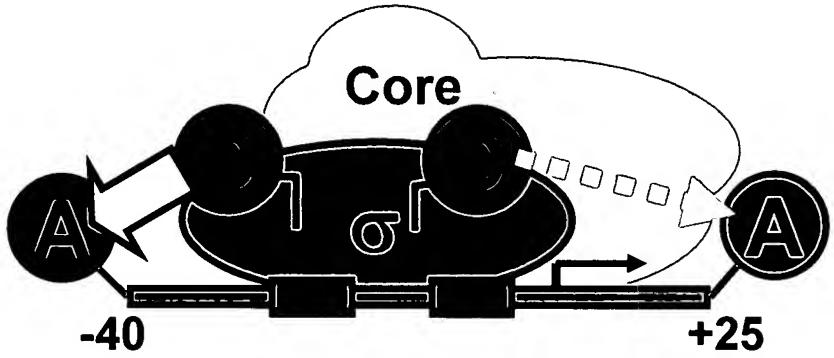


Funding: DOE, NIH

TRAILING-EDGE and LEADING-EDGE FRET:

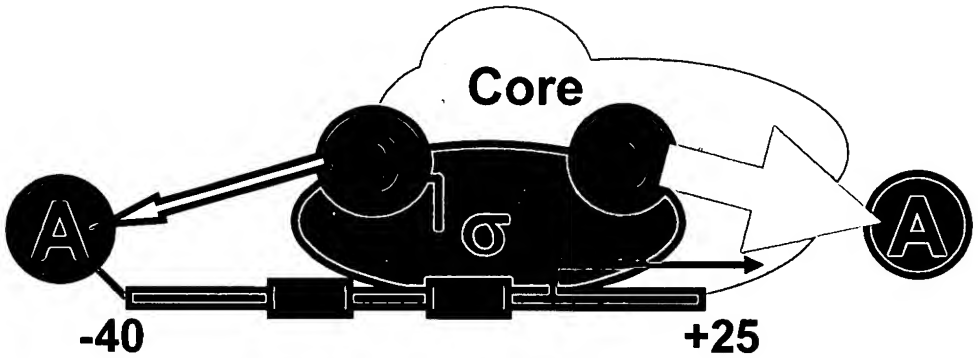
Assay of translocation of a protein relative to a nucleic acid

Trailing-edge/leading-edge FRET (TELE-FRET)



Ruler 1: High FRET

Ruler 2: Low FRET

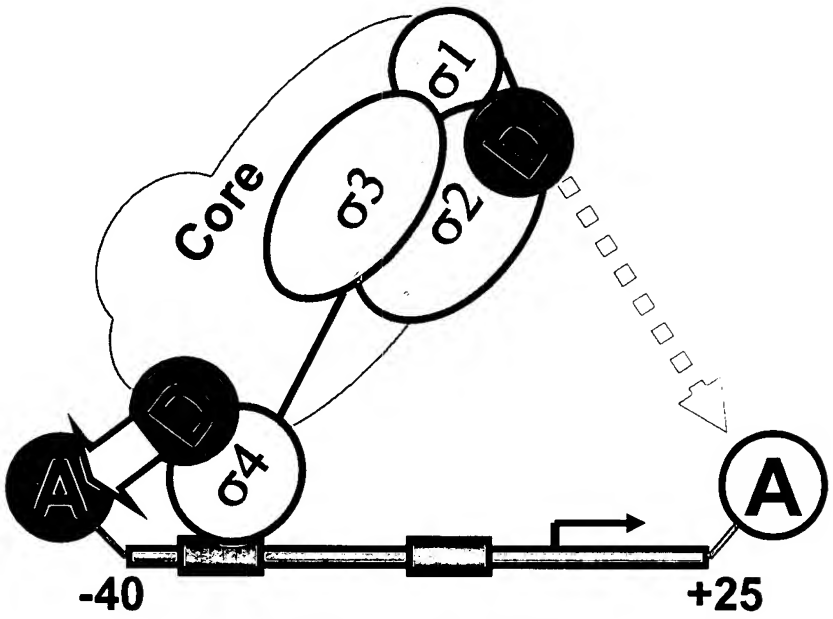


Ruler 1: Low FRET

Ruler 2: High FRET

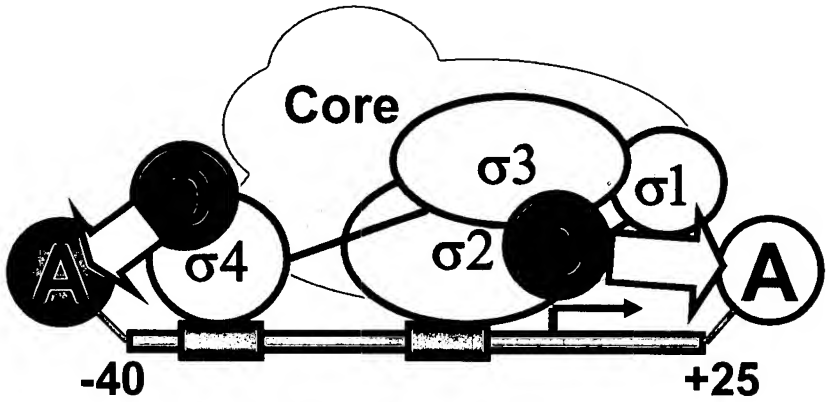
Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

Step-Sequence of formation of promoter contacts using 2 FRET rulers



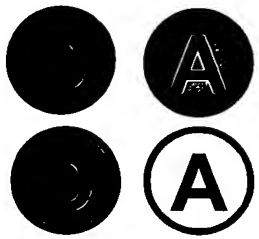
Ruler 1: High FRET

Ruler 2: Low FRET



Ruler 1: High FRET

Ruler 2: High FRET



Ruler 1

Ruler 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Shimon Weiss	Art Unit: 2877
Appl. No.: 10/561,448	Examiner: F.L. Evans
Confirmation No.: 8178	
Filed: December 20, 2005	Atty. Docket No.: 58086-226455
For: MODULATED EXCITATION FLUORESCENCE ANALYSIS	Customer No. 26694
	PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 C.F.R. § 1.131

Honorable Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, being duly warned, declare the following:

1. I am a co-inventor of the subject matter described and claimed in the above-identified U.S. patent application. I have reviewed the claims of this application as currently amended.

2. I understand that the Office Action dated November 30, 2007 rejected the examined claims of this patent application under 35 U.S.C. § 102(a) over published German patent application Publication No. DE 10210737 A1 by Krieger et al. that published March 20, 2003.

3. I, together with my co-inventors, conceived the invention described and claimed in at least independent claims 1 and 21 of this application, and reduced it to practice, prior to the March 20, 2003 publication date of the cited reference. Our prior invention is evidenced by a copy of a presentation by one of the co-inventors, Achillefs Kapanidis, at the Single-Molecule Biophysics Conference in Aspen, CO on January 7, 2003, (copy attached as Exhibit A).

4. As documented by Exhibit A, my co-inventors and I conceived the invention of at least current independent claims 1 and 21, and reduced it to practice, prior to January 7, 2003.

5. The acts described above in paragraphs 3 and 4 were carried out in the United States of America, or else in a WTO member country.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

05/28/08
Date

Shimon Weiss
Shimon Weiss

Date

Achillefs Kapanidis

Date

Ted A. Laurence

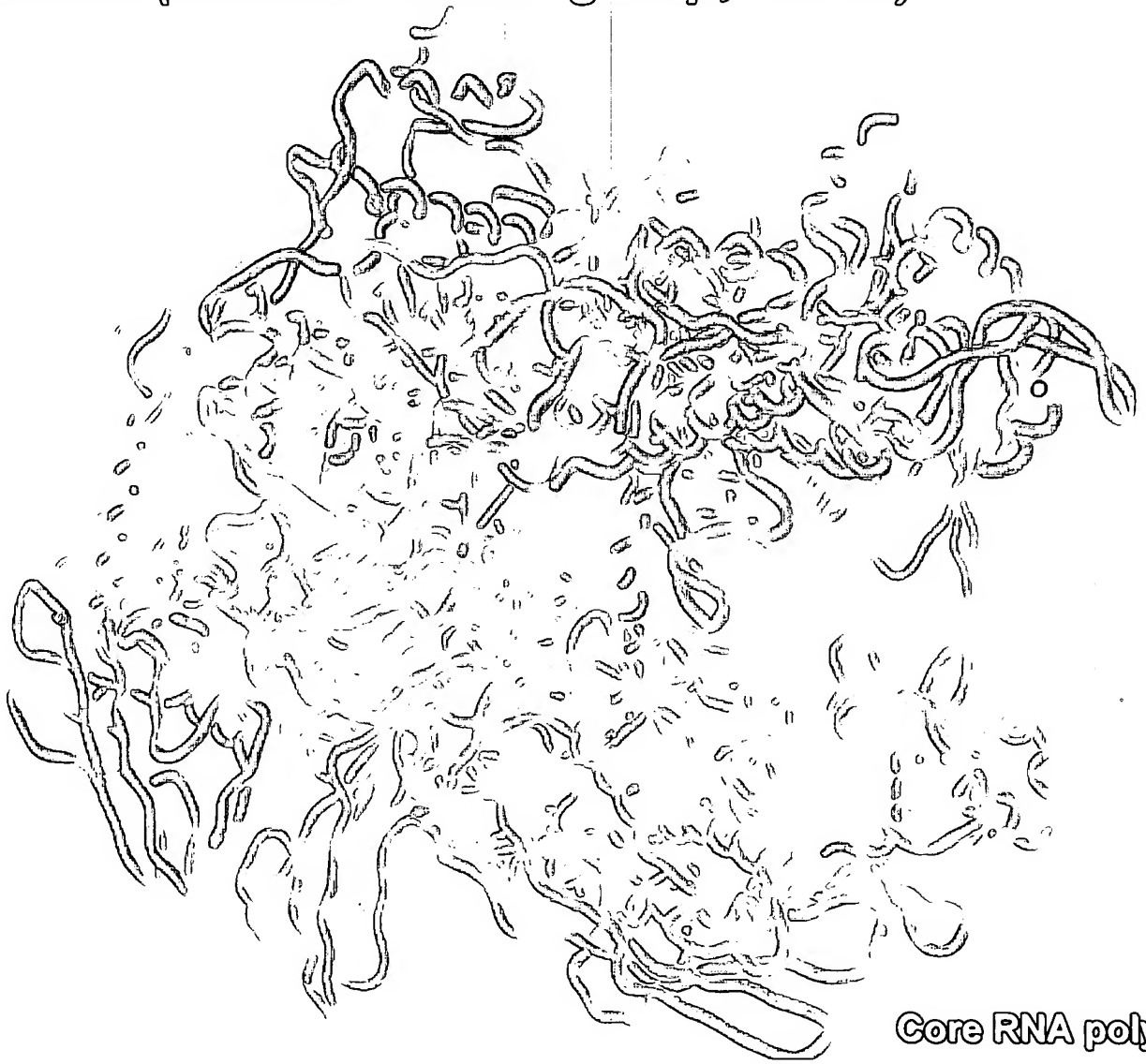
Date

Nam K. Lee

Exhibit A

Molecular Machines at Work:
Single-Molecule Analysis of Transcription by RNA Polymerase

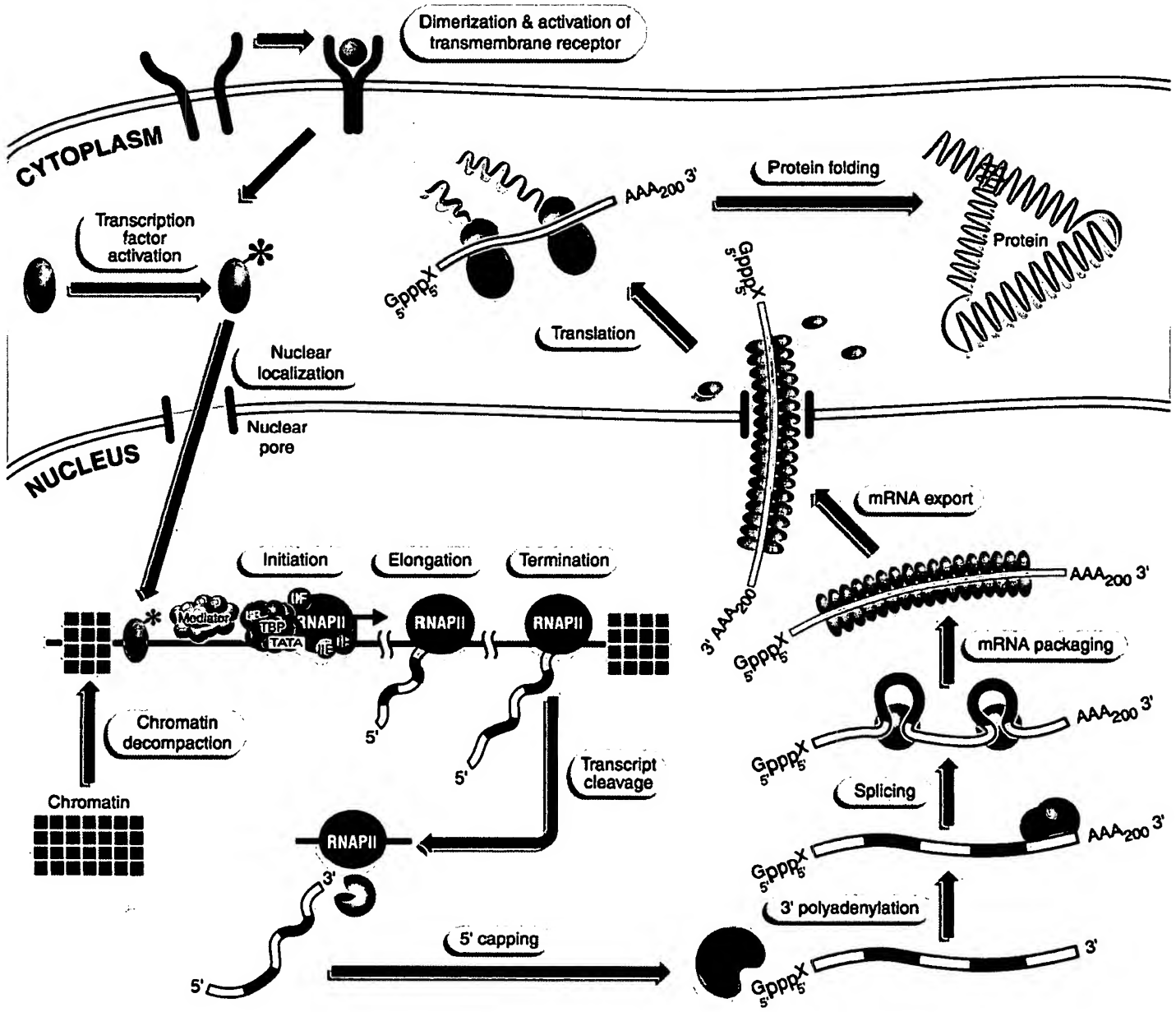
Achillefs Kapanidis (Shimon Weiss' group, UCLA)



Core RNA polymerase (Darst lab)

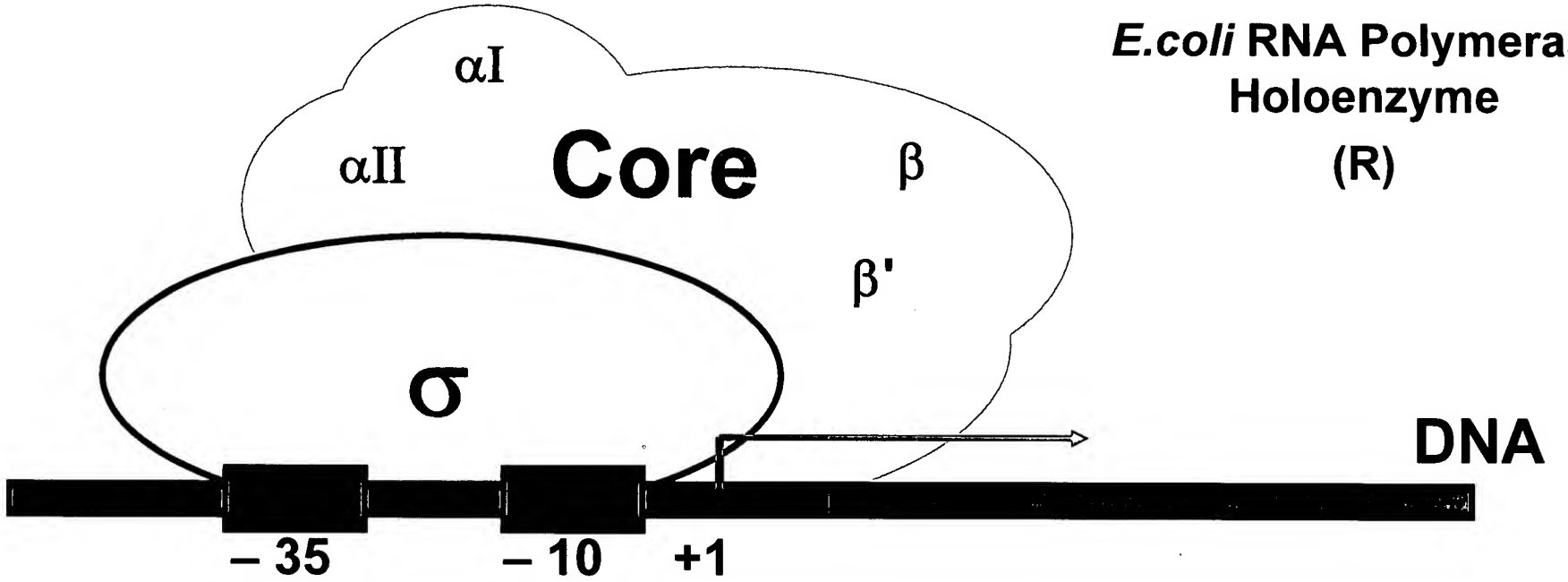
Single-Molecule Biophysics Conference: Aspen, Jan.7, 2003

GENE EXPRESSION:
The path from gene to protein

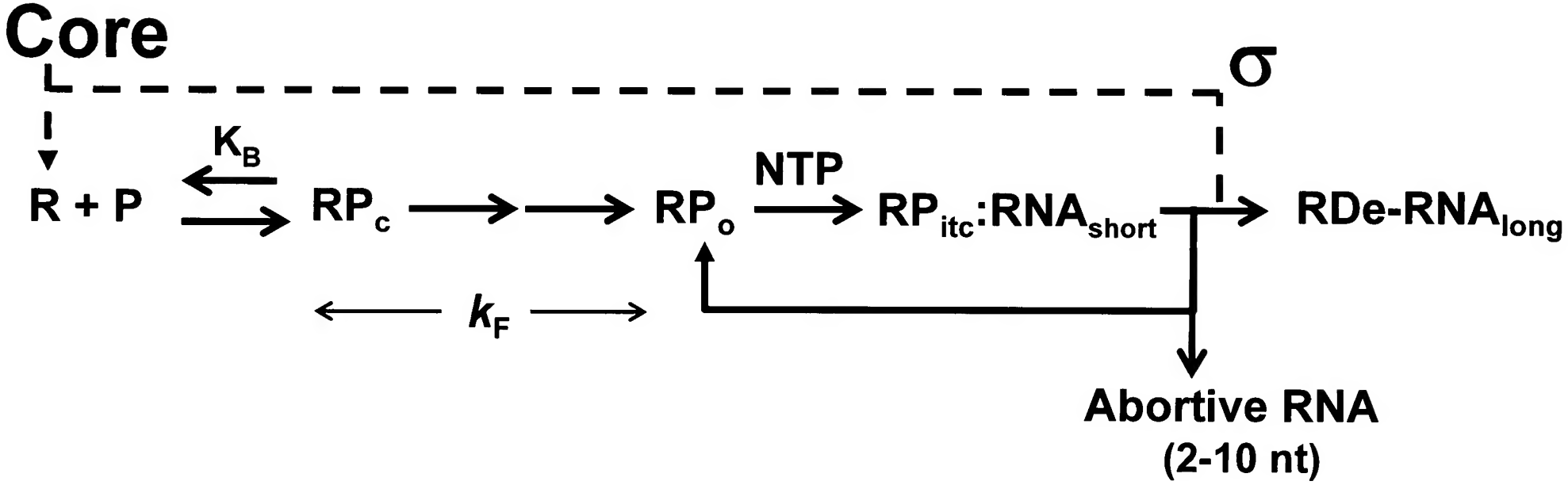


Orphanides and Reinberg, 2002

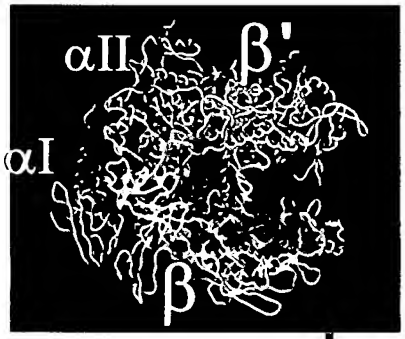
TRANSCRIPTION INITIATION



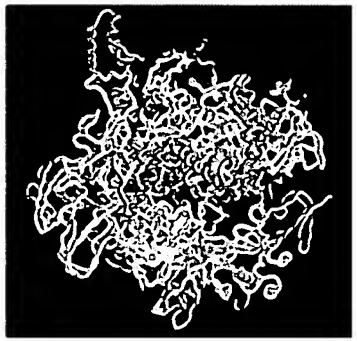
E.coli RNA Polymerase Holoenzyme (R)

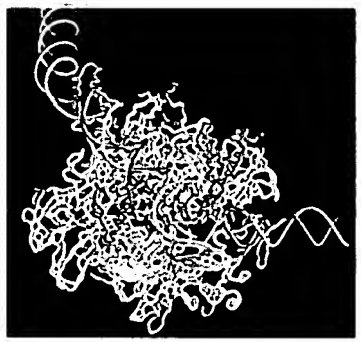


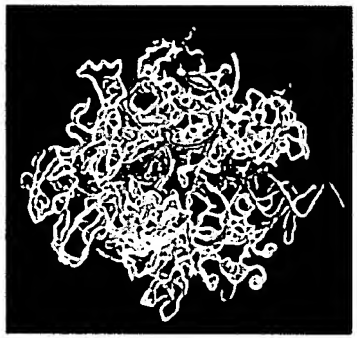
STRUCTURAL ASPECTS OF TRANSCRIPTION



RNAP core ($\alpha_2\beta\beta'$)


RNAP holoenzyme (core + σ)


RP_o


RD_e (model)

X-ray structures → static snapshots of the machine

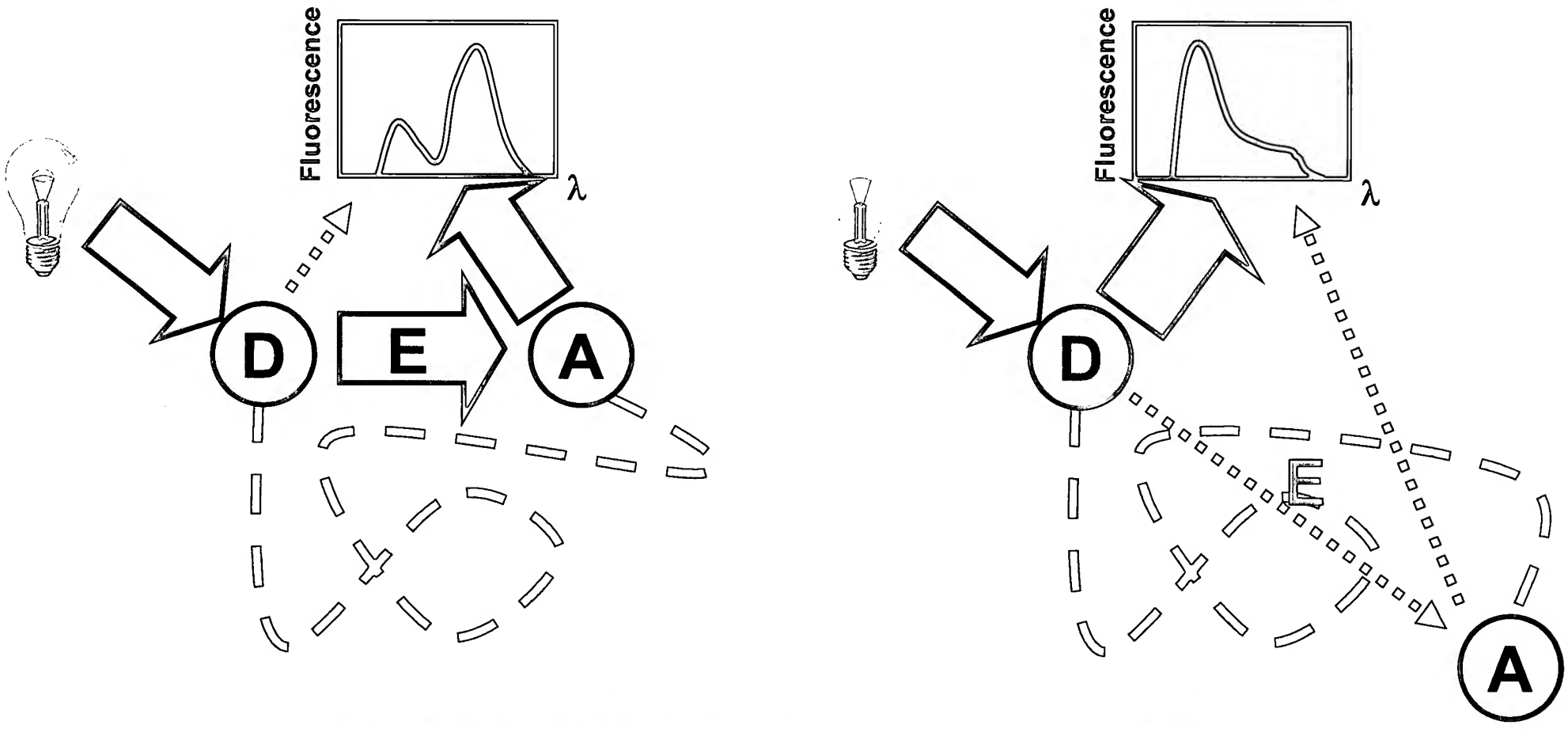
SMD: "movie" of the dynamic process

Structure
Dynamics
Local Environment → **Intermediates** **Kinetics** → **Timing** **of Events** → MECHANISM

Young *et al.*, 2002

FÖRSTER RESONANCE ENERGY TRANSFER (FRET):

A "MOLECULAR RULER" FOR THE 2-10 nm REGIME



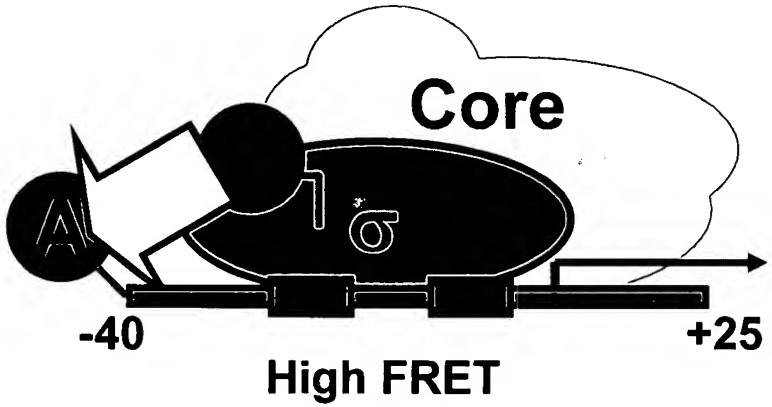
FRET Efficiency, $E = [1 + (R/R_o)^6]^{-1}$

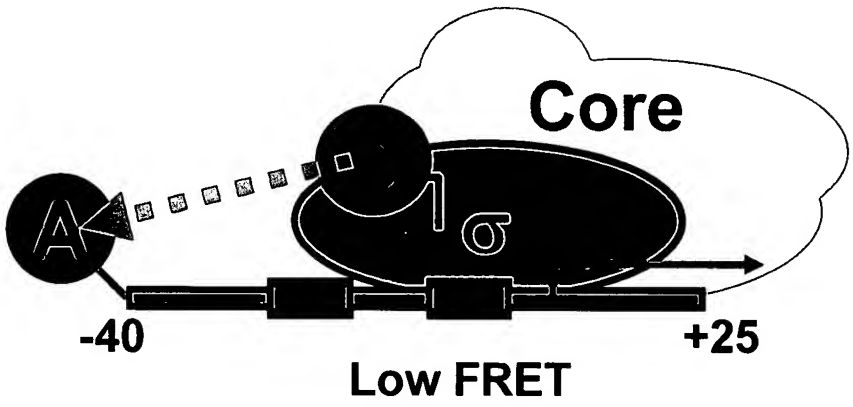
R = D-A Distance

TRAILING-EDGE and LEADING-EDGE FRET:

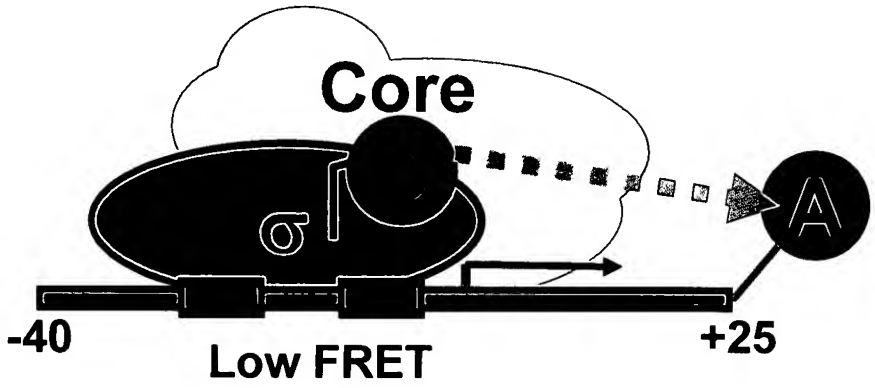
Assay of translocation of a protein relative to a nucleic acid

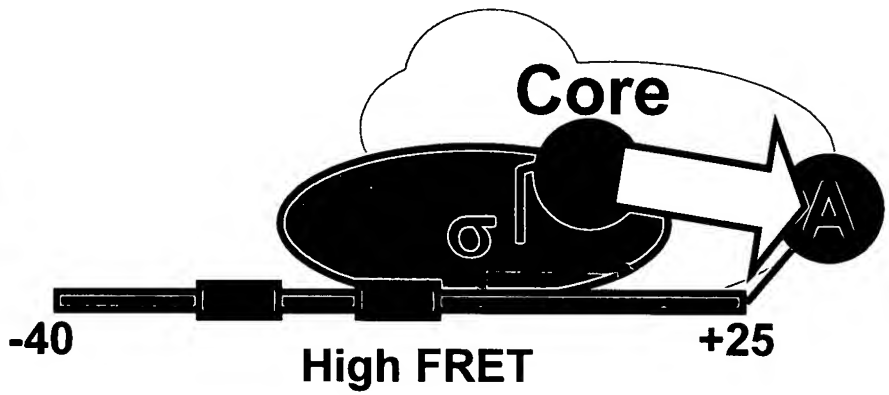
Trailing-edge FRET





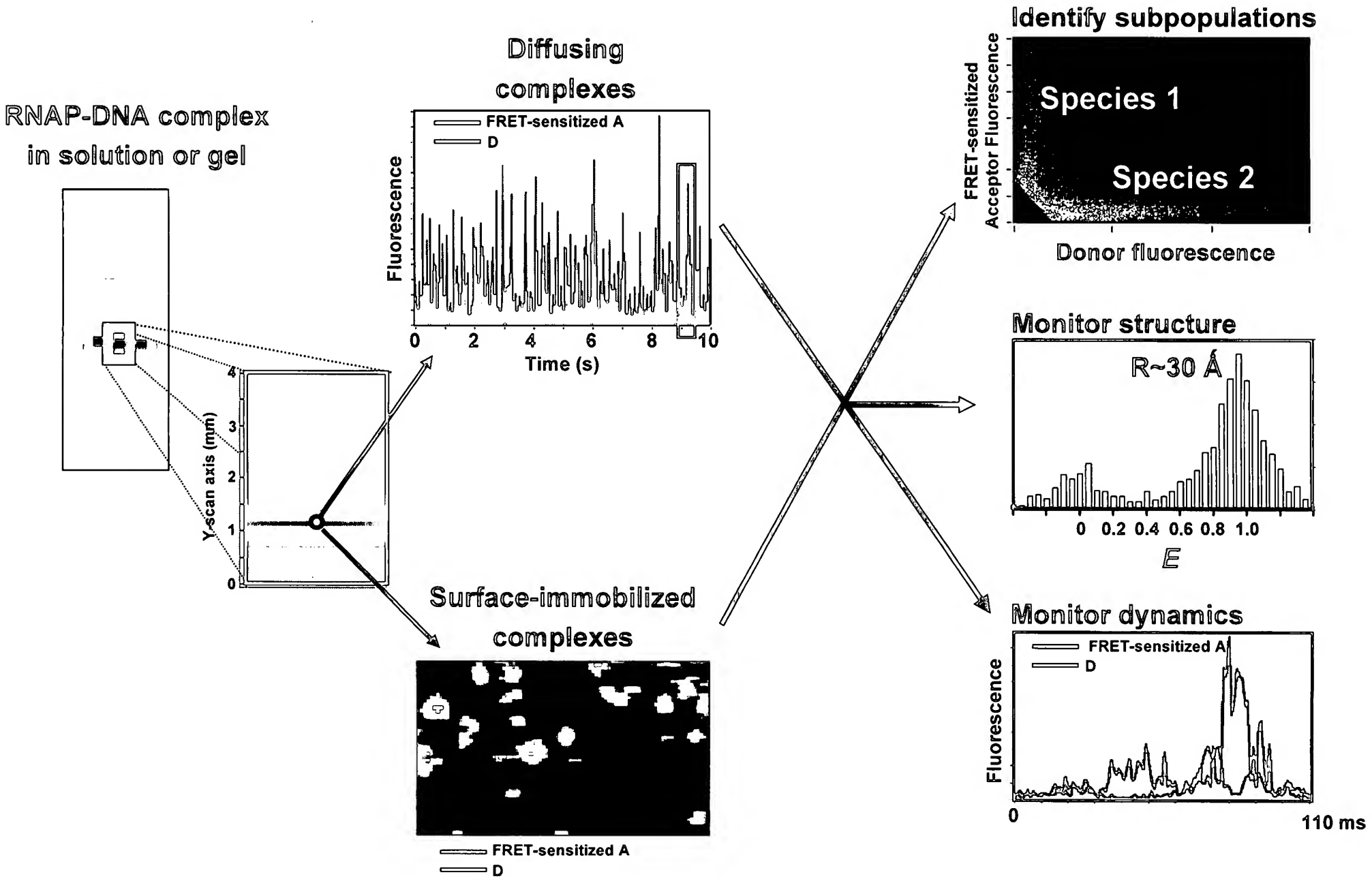
Leading-edge FRET





Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

sp-FRET ON RNAP-DNA COMPLEXES

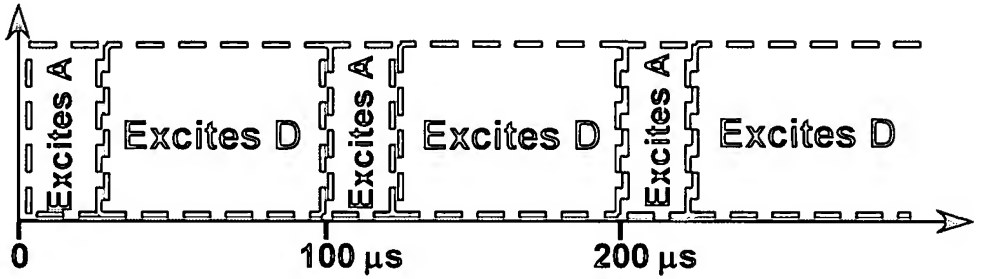


LIMITATIONS OF SINGLE-LASER EXCITATION spFRET

- Complex FRET Acceptor photophysics
  - "Dark" states→D-only peak
  - Photobleaching→ D-only peak
  - Intermittency ("Blinking")
- Complex FRET Donor photophysics
  - Intermittency
  - Transient QY changes
- Limited discrimination ability in the FRET coordinate
  - FRET range of 0-0.3 not easily accessible
- Variable fluorescence contamination
  - Adds variable counts to D-only peak

sp-FRET USING ALTERNATE LASER EXCITATION (ALEX)

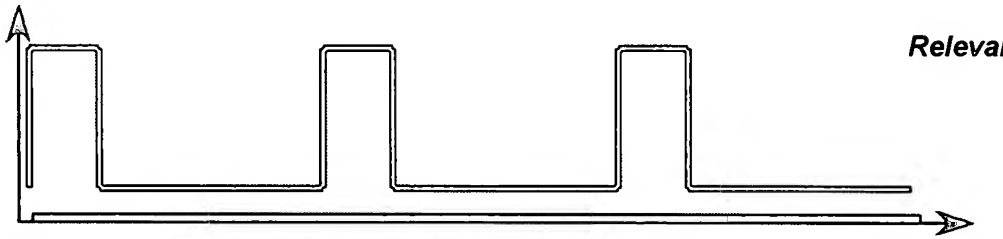
EXCITATION PROFILE



EMISSION PROFILES

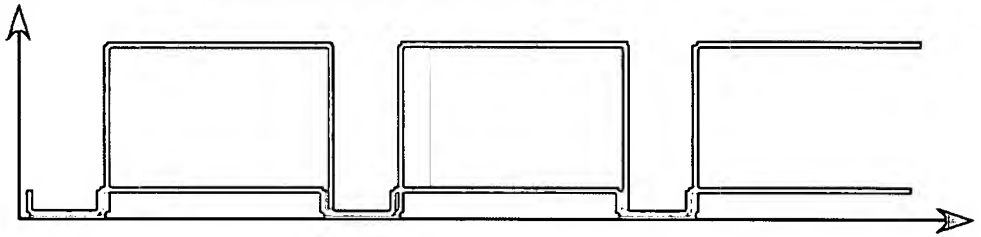
Relevant transcription species

A-only species



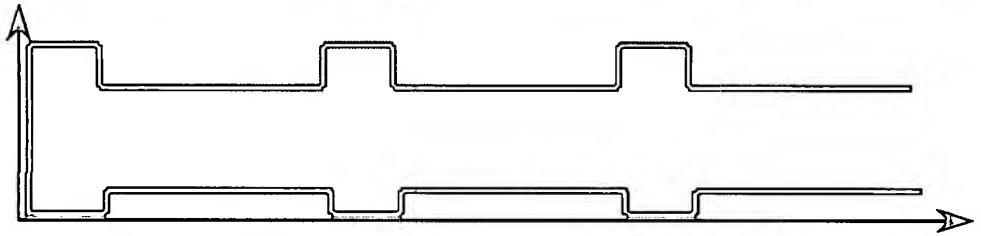
DNA^{A}

D-only species



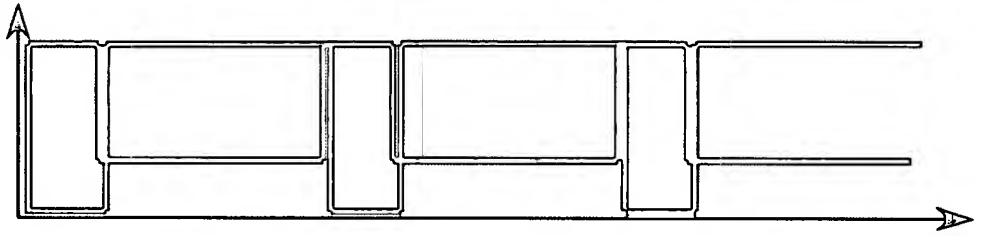
$RNAP\sigma^{D}$

D-A species, high FRET



RPo^{D-A}, Trailing-edge

D-A species low FRET



RPo^{D-A}, Leading-edge

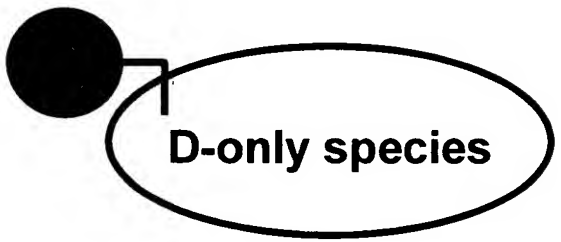
EQUATIONS

Energy transfer ratio (*E*)

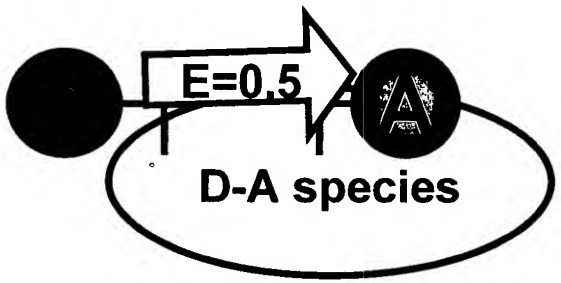
$$E = \frac{F^{DA}_{670em,514ex}}{F^{DA}_{670em,514ex} + F^{DA}_{580em,514ex}}$$

ALEX-based ratio (*ALEX*)

$$ALEX = \frac{F_{514ex}}{F_{514ex} + F_{638ex}} = \frac{F_{670em,514ex} + F_{580em,514ex}}{F_{670em,514ex} + F_{580em,514ex} + F_{670em,633ex}}$$

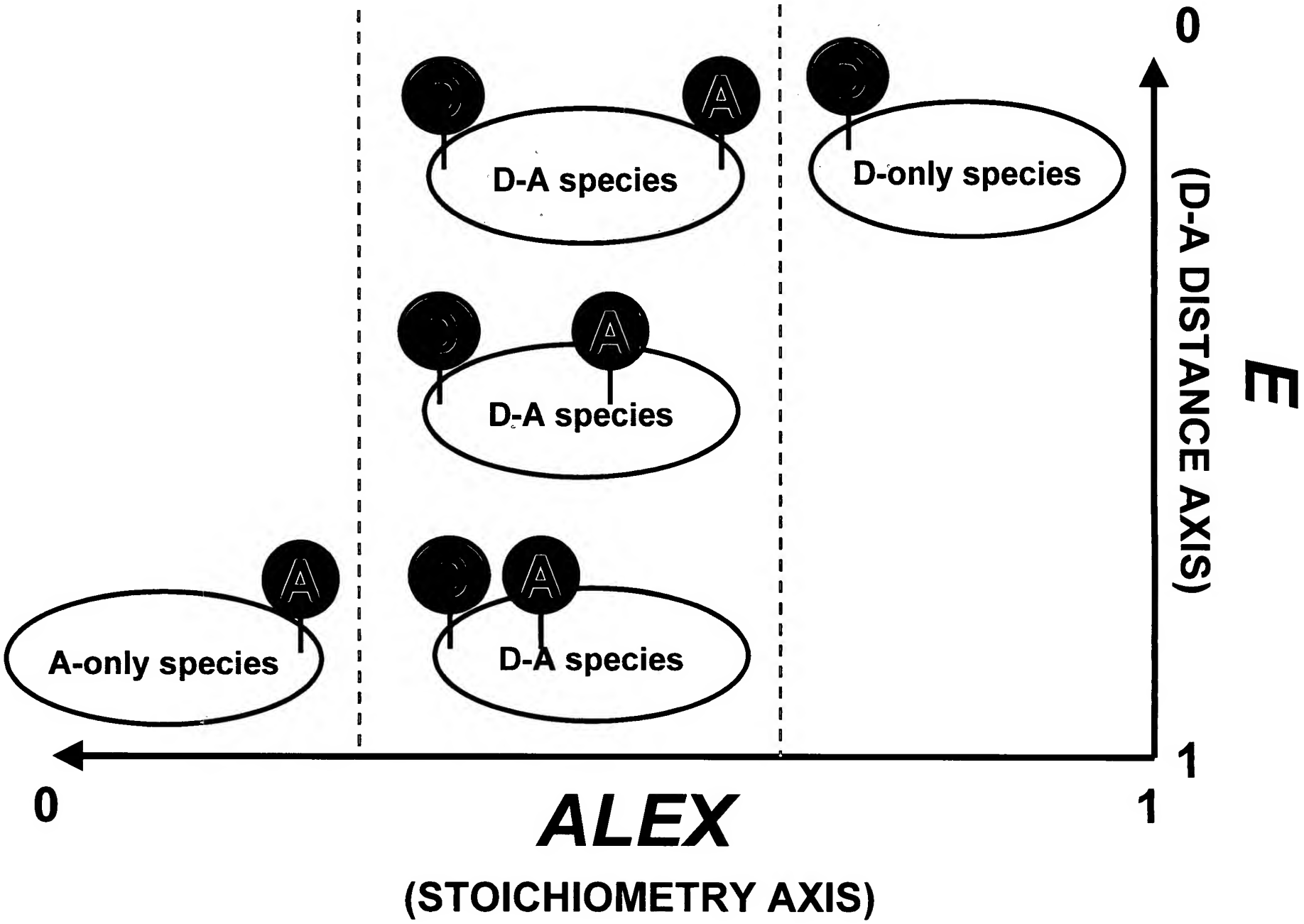


$$ALEX = \frac{0+100}{0+100+0} \sim 1.0$$

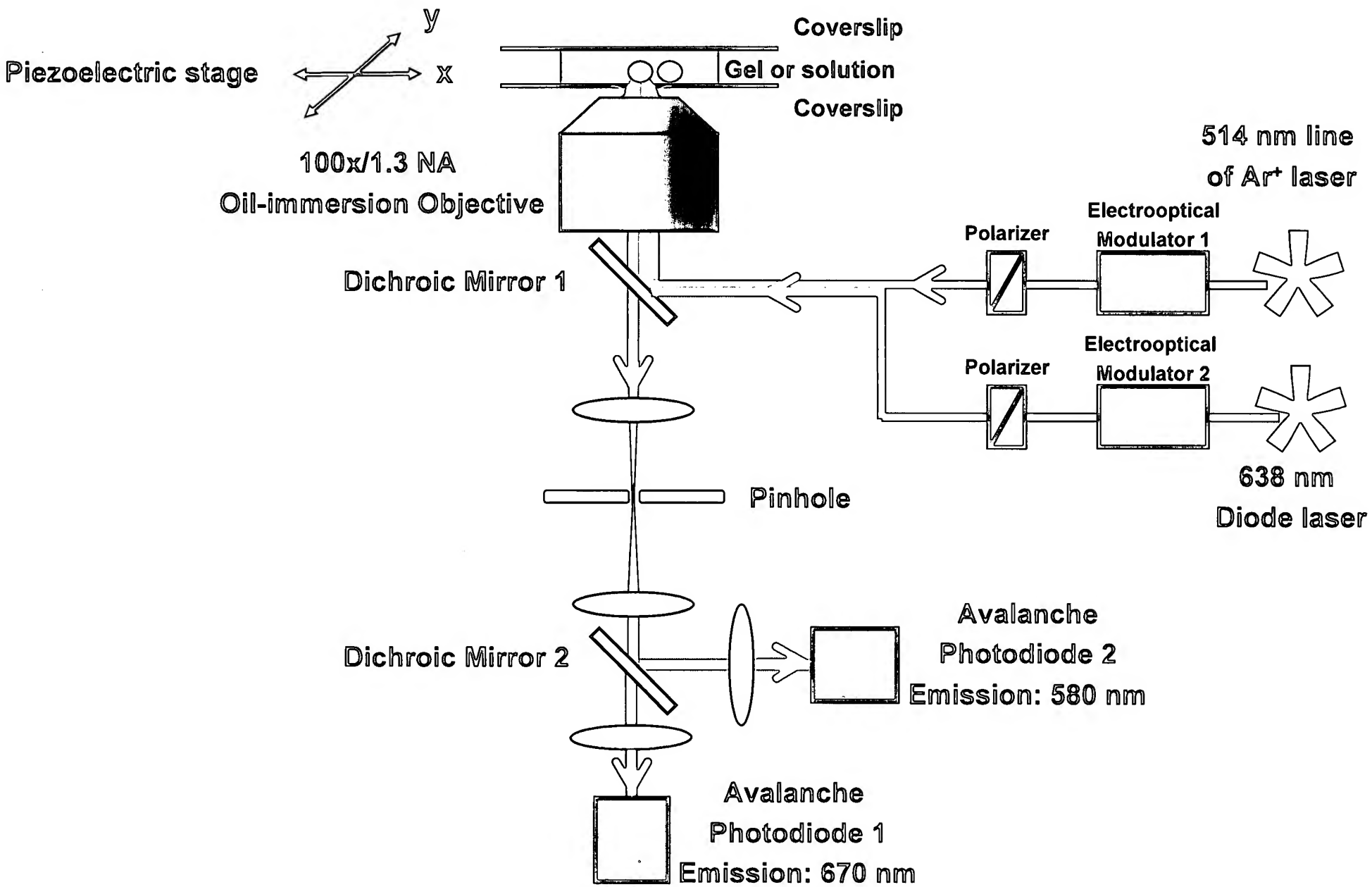


$$ALEX = \frac{50+50}{50+50+100} \sim 0.5$$

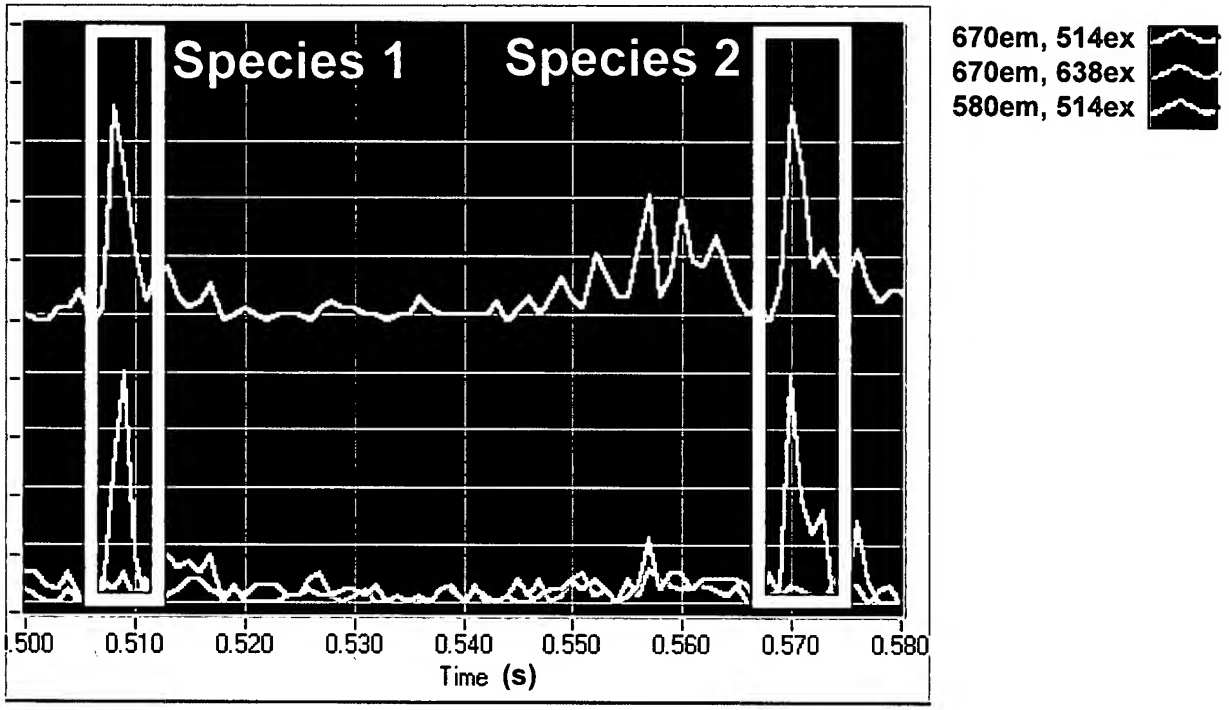
$$ALEX = \frac{0+0}{0+0+100} \sim 0.0$$


SORTING SPECIES USING E, ALEX
D-A species
D-only species
D-A species
A-only species
D-A species
0
1
E
(D-A DISTANCE AXIS)
0
1
ALEX
(STOICHIOMETRY AXIS)

ALEX SINGLE-MOLECULE CONFOCAL MICROSCOPY

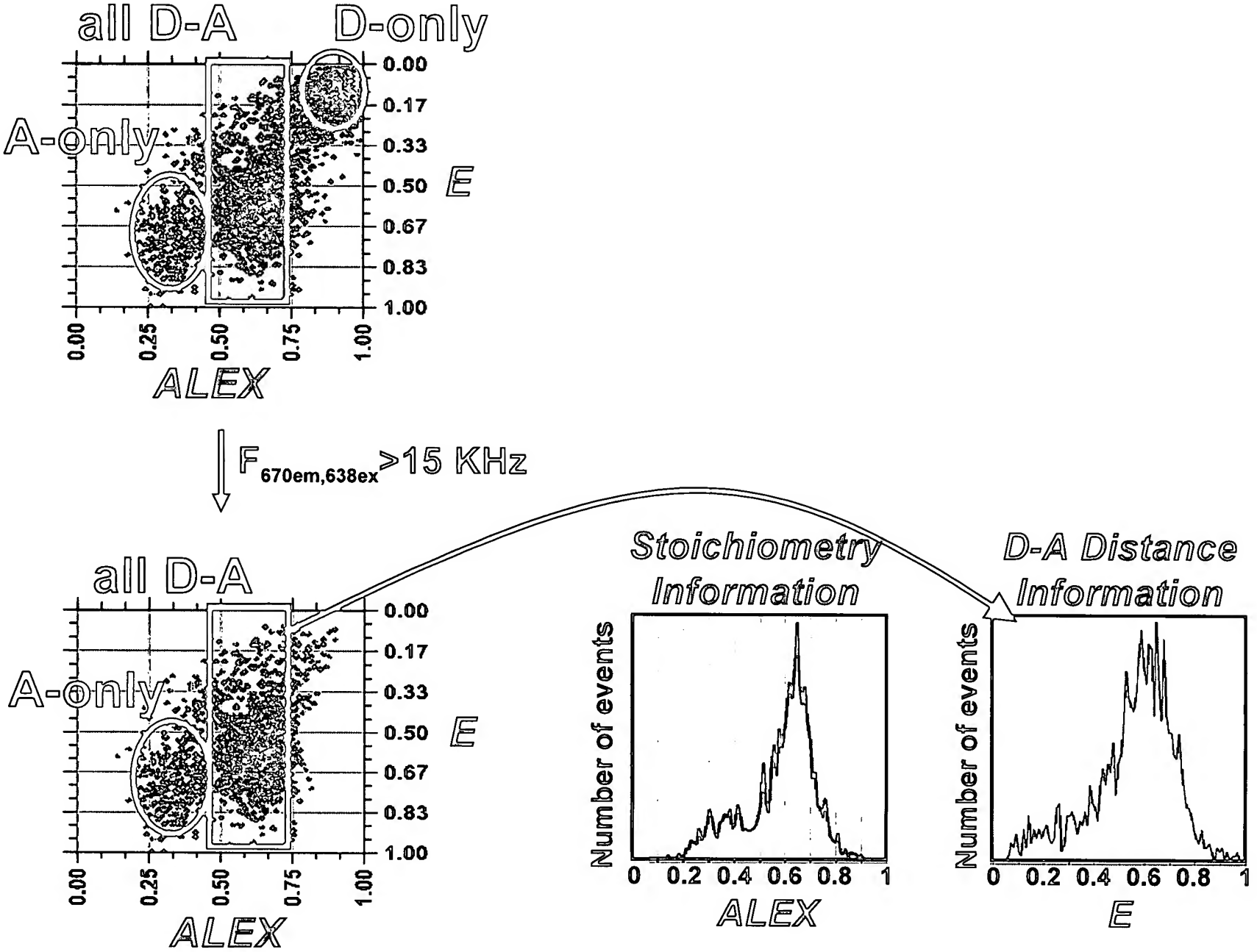


DATA ANALYSIS FOR INDIVIDUAL SPECIES

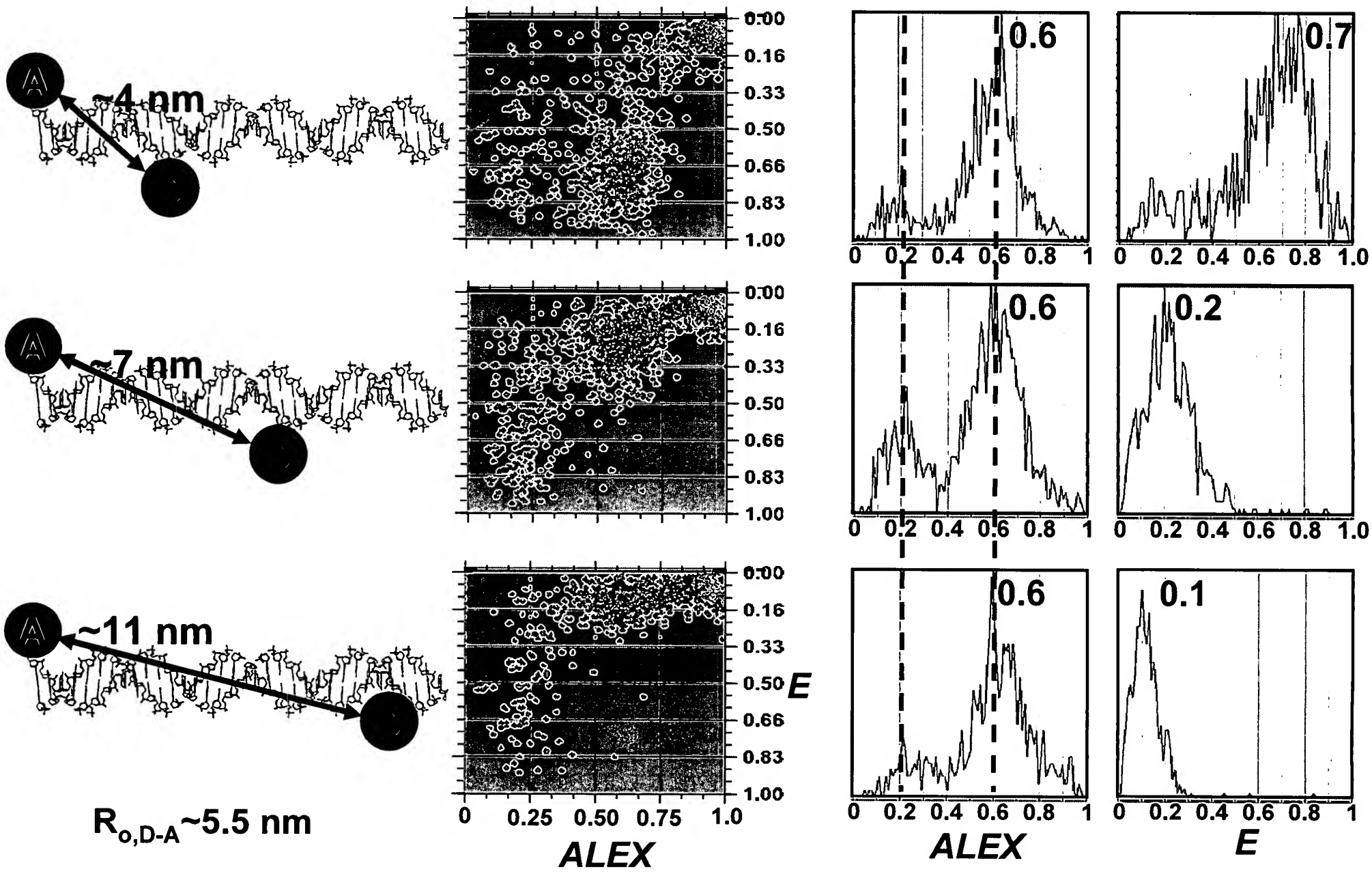


	Species 1	Species 2
670em, 514ex	71	85
670em, 638ex	69	93
580em, 514ex	7	11
FRET-sensitized A	52	60
E, simplified	91%	88%
E, FRET-sensitized A	91%	77%
ALEX	0.49	0.66

DATA ANALYSIS USING *E-ALEX* 2-D HISTOGRAMS



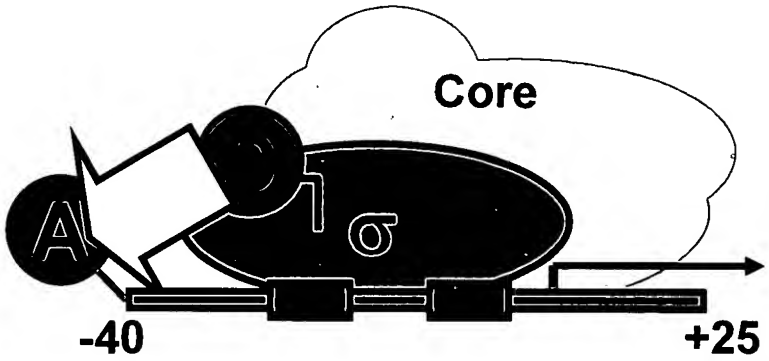
MODEL SYSTEMS: dsDNA



$R_{o,D-A}$~5.5 nm

USING TRAILING-EDGE sp-FRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE

OPEN COMPLEX

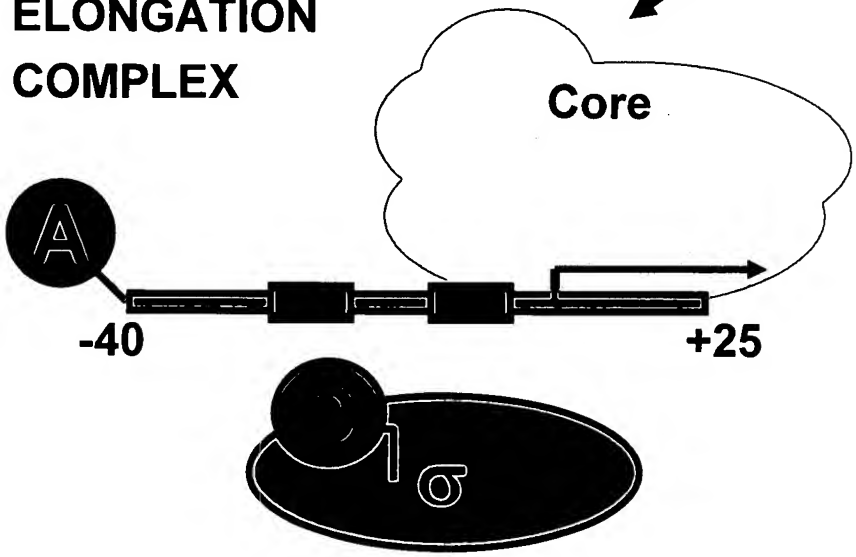


D and A co-localize; High E

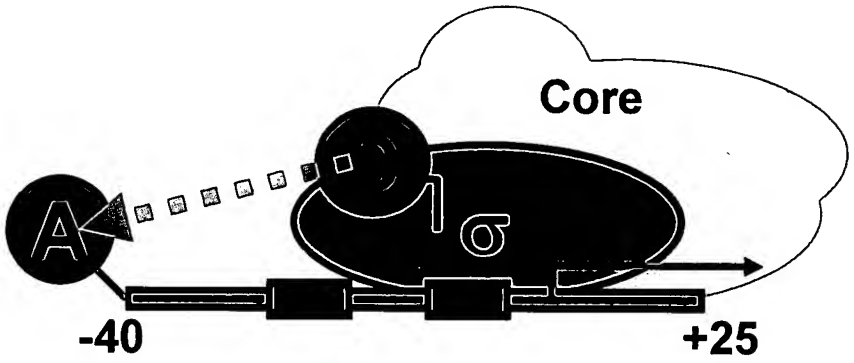
σ release model

σ non-release model

ELONGATION COMPLEX



D and A do *not* co-localize; Zero E



D and A co-localize; Zero or low E

Mukhopadhyay *et al.*, 2001

TRAILING-EDGE spFRET

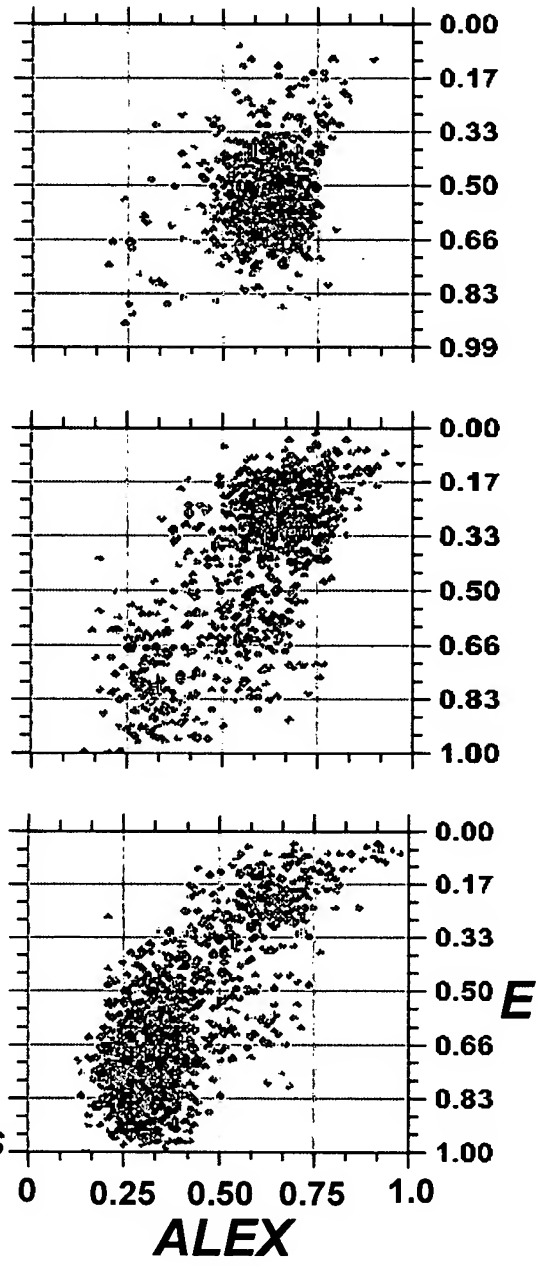
RNAP$\sigma^{TMR,569}$→lacUV5-11$^{Cy5,-40}$

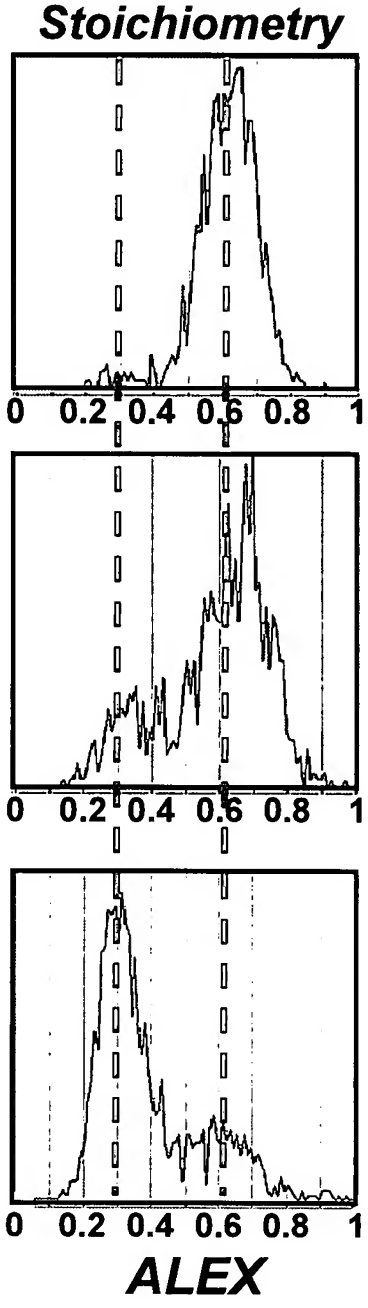
RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

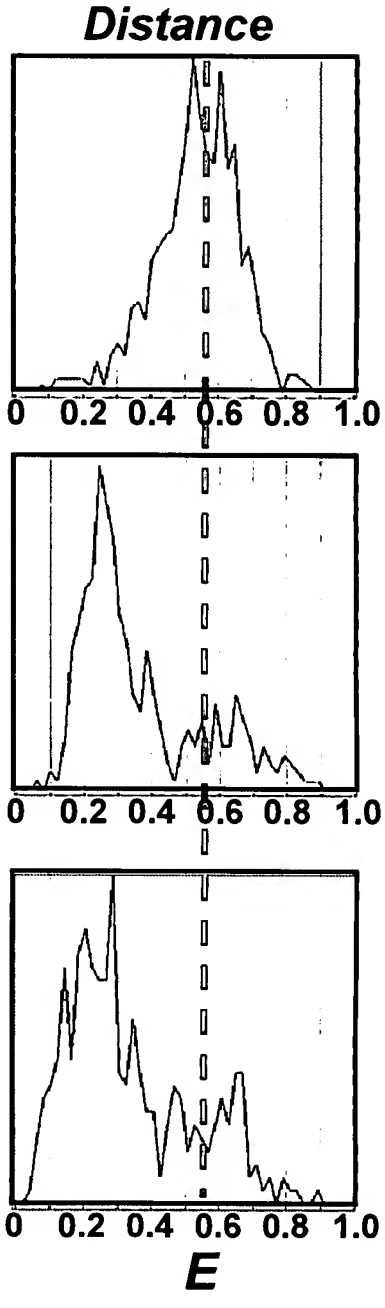
RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

RPo + ApA
+ 60 μM NTPs
(chase)

ABILITY OF STALLED COMPLEXES TO RESUME TRANSCRIPTION

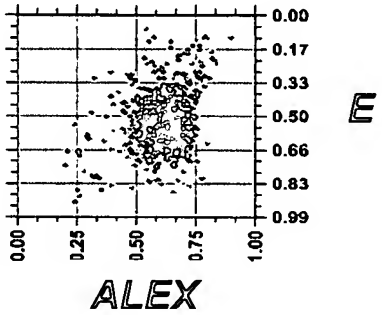




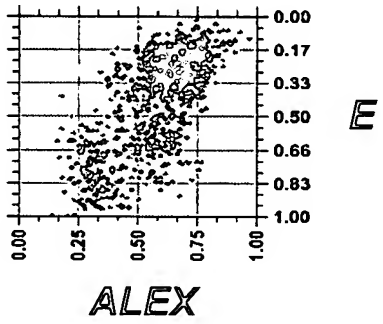


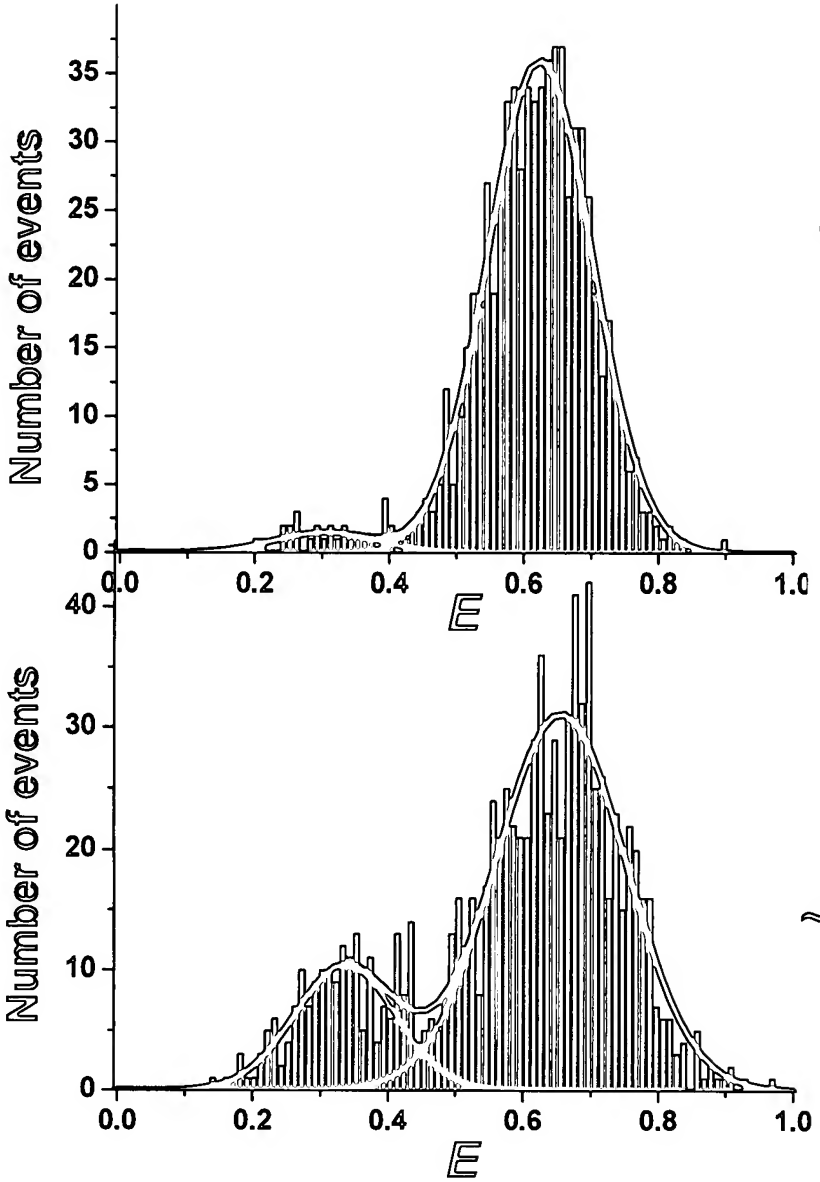
DIRECT OBSERVATION OF SIGMA NON-RELEASE: TRAILING-EDGE spFRET

$RP_{itc,2}$



$RD_{e,11}$





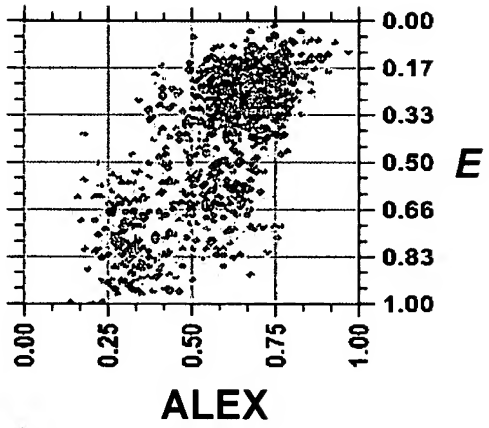
~5% dissociation

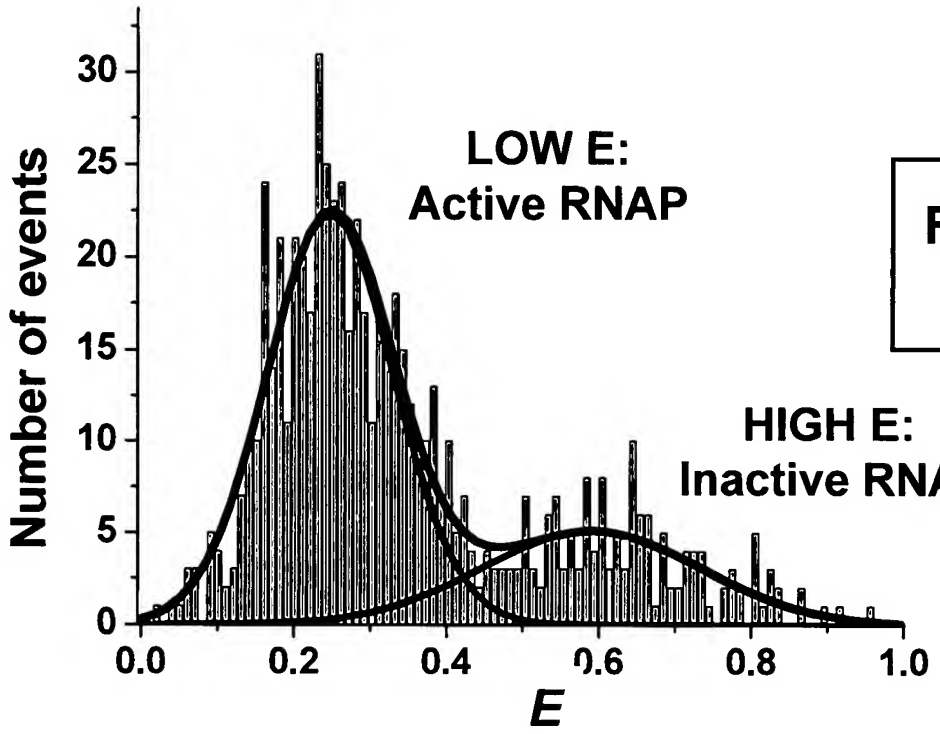
~20% dissociation

$$\% \text{ Dissociation} = \frac{(\text{A-only})}{(\text{A-only})+(\text{all D-A})}$$

E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: TRAILING-EDGE spFRET

RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

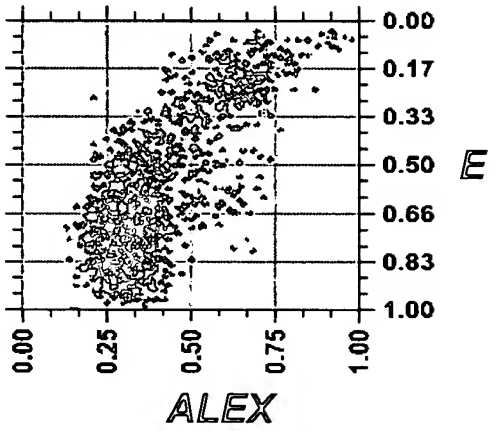


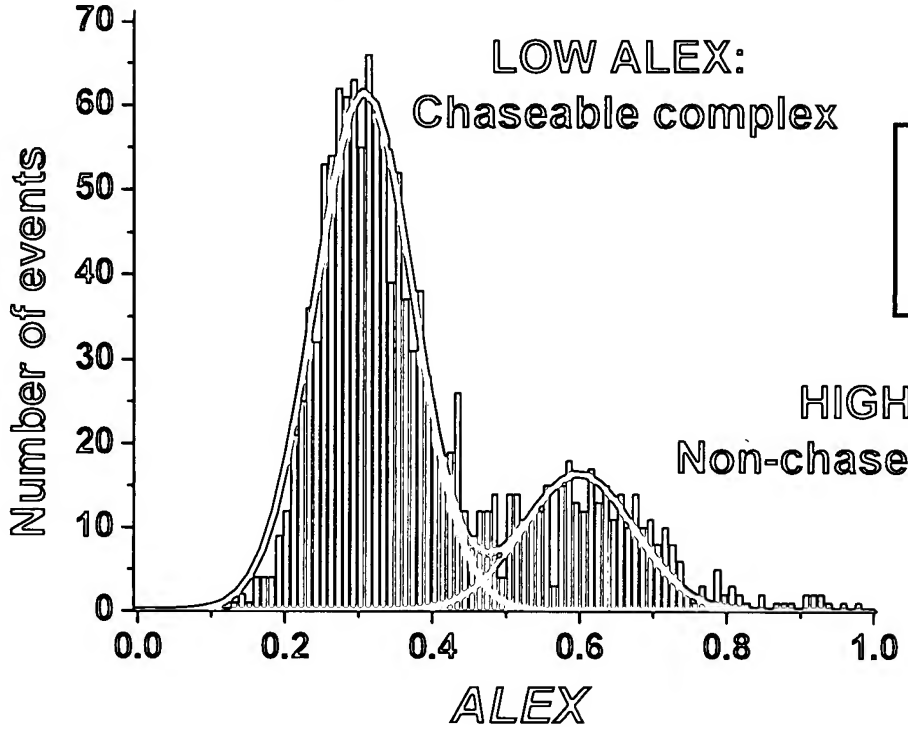


RNAP translocational activity ~ 70%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": TRAILING-EDGE spFRET

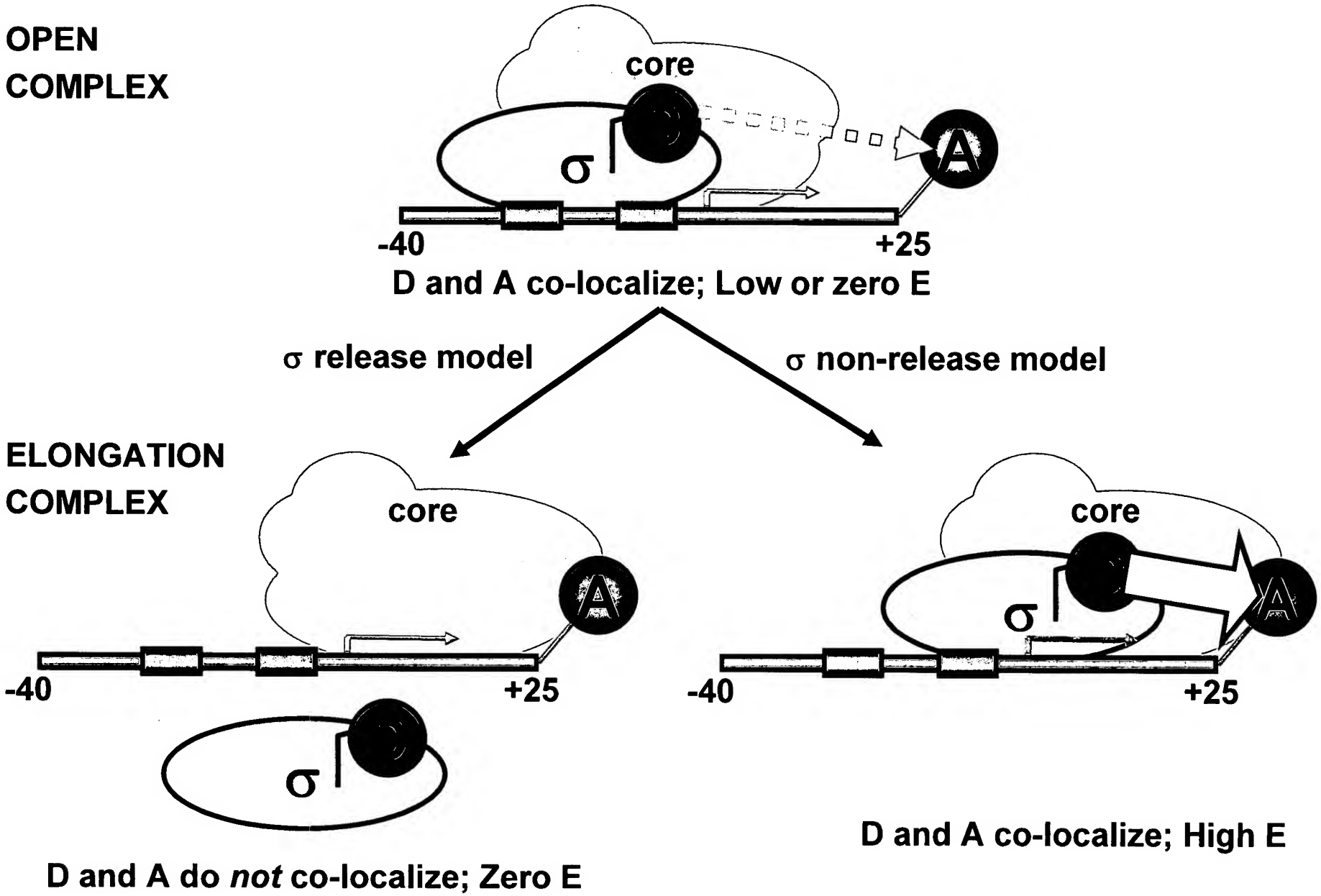
RPo + ApA + 60 μM NTPs (chase)





RNAP "chaseability"
~ 80%

USING LEADING-EDGE spFRET TO ANALYZE SIGMA RELEASE UPON PROMOTER ESCAPE



Mukhopadhyay *et al.*, 2001

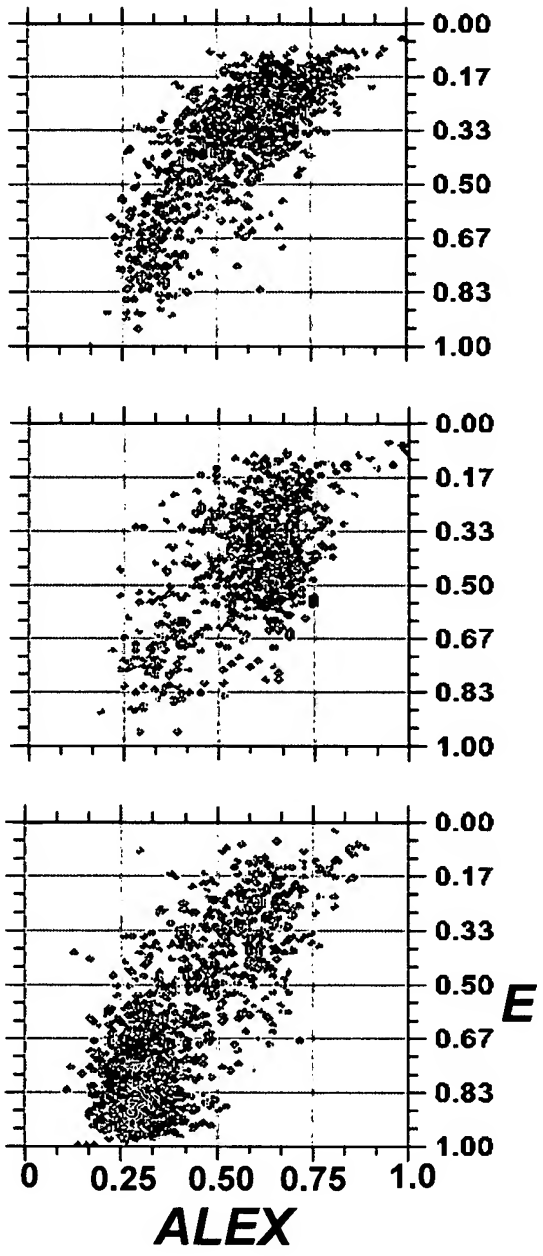
LEADING-EDGE spFRET

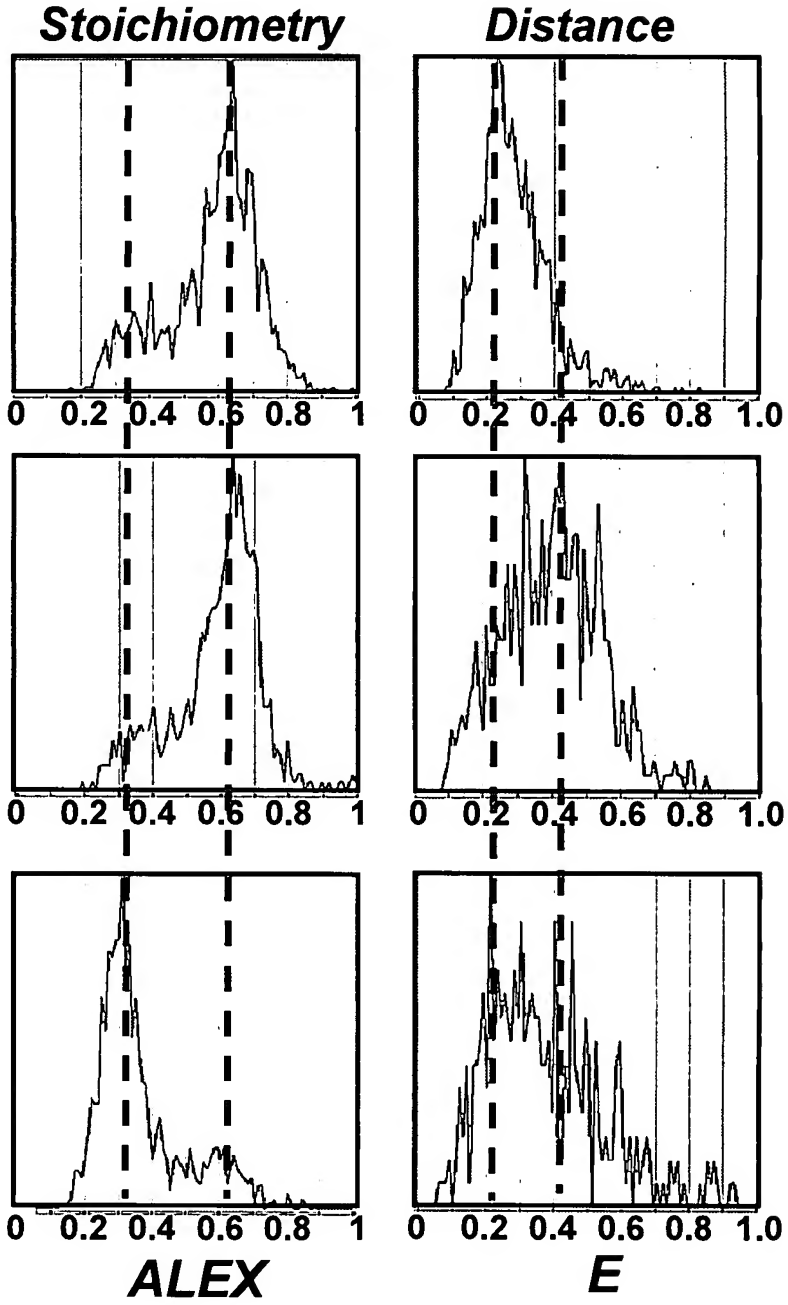
$RNAP\sigma^{TMR,366} \rightarrow lacUV5\text{-}11^{Cy5,+25}$

RPo + ApA
($RP_{itc,2}$)
(equivalent to RPo)

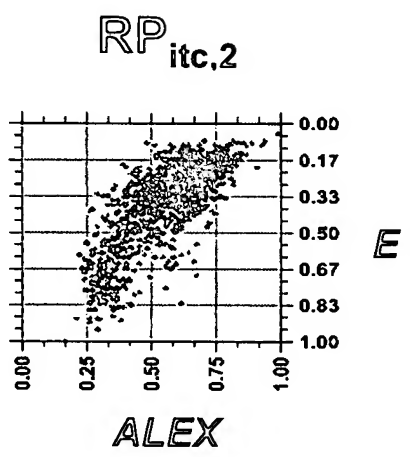
RPo + ApA
+ 12.5 μM UTP/GTP/ATP
($RD_{e,11}$)

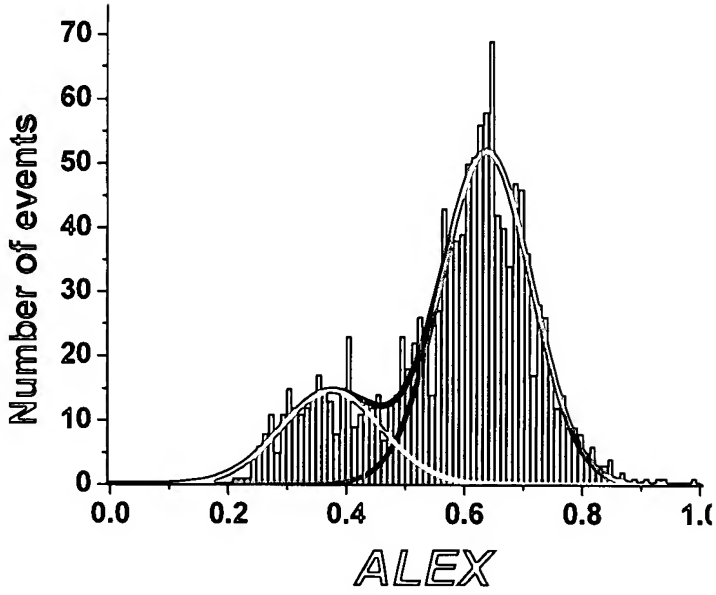
RPo + ApA
+ 60 μM NTPs
(chase)



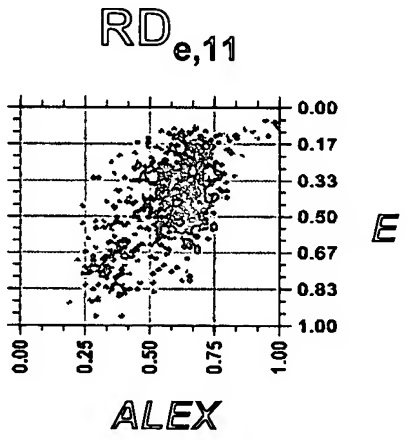


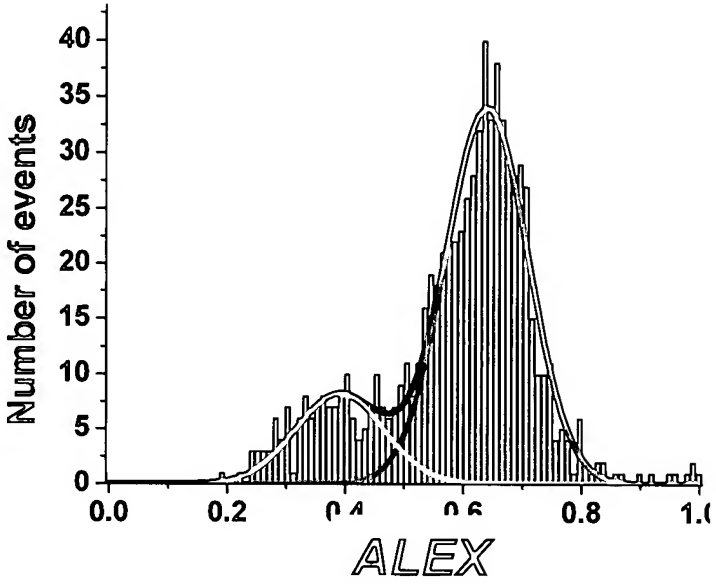
DIRECT OBSERVATION OF SIGMA NON-RELEASE: LEADING-EDGE spFRET





~20% dissociation

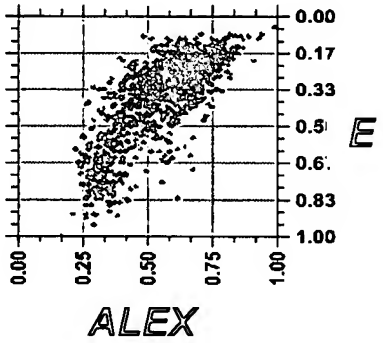


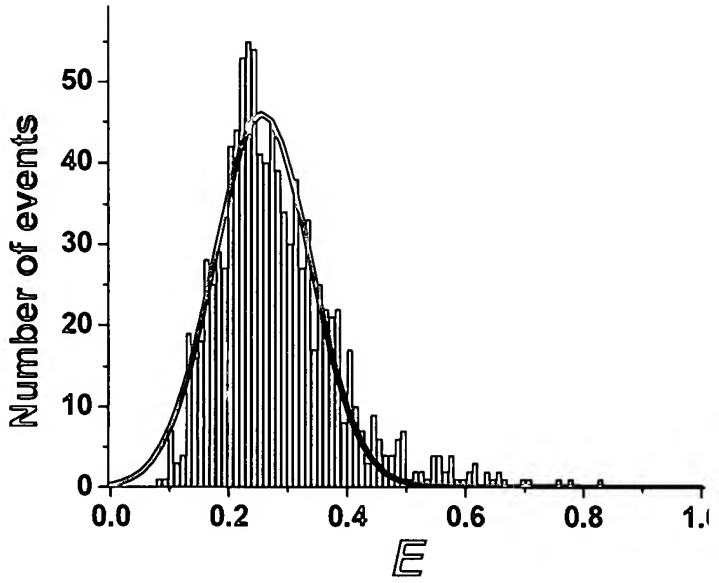


~20% dissociation

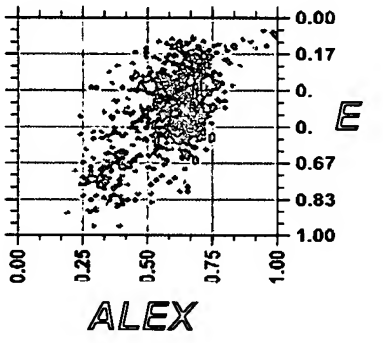
E HISTOGRAM MONITORS ABILITY OF RNAP TO TRANSLOCATE UPON ESCAPE: LEADING-EDGE spFRET

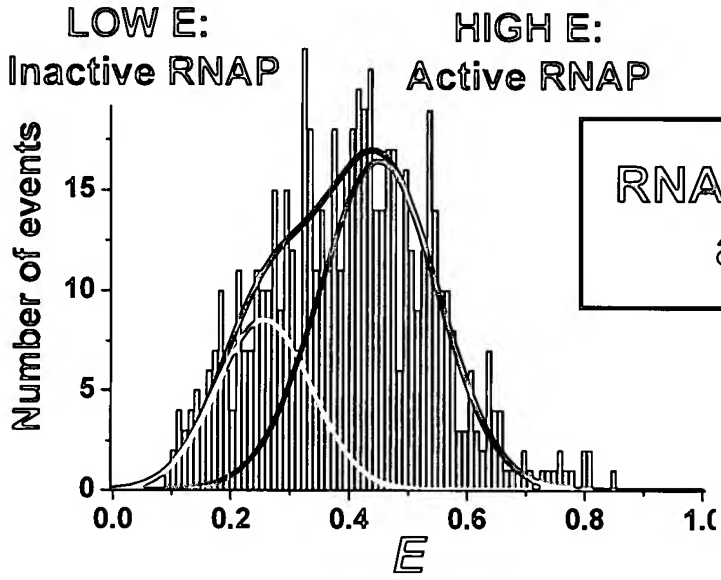
RPo + ApA ($RP_{itc,2}$)





RPo + ApA + 12.5 μM UTP/GTP/ATP ($RD_{e,11}$)

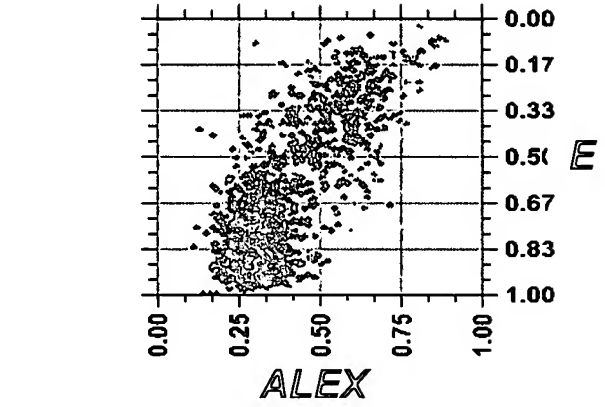


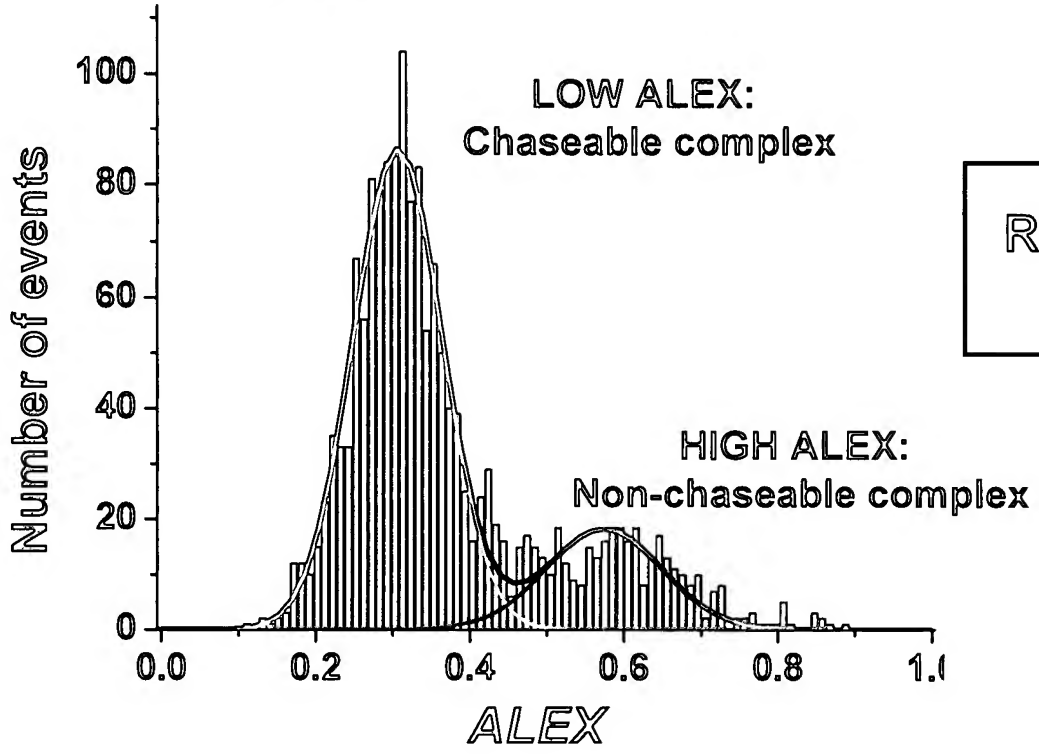


RNAP translocational activity = 72%

DISSOCIATION HISTOGRAM MONITORS ABILITY OF RNAP TO BE "CHASED": LEADING-EDGE spFRET

RPo + ApA + 60 μM NTPs (chase)

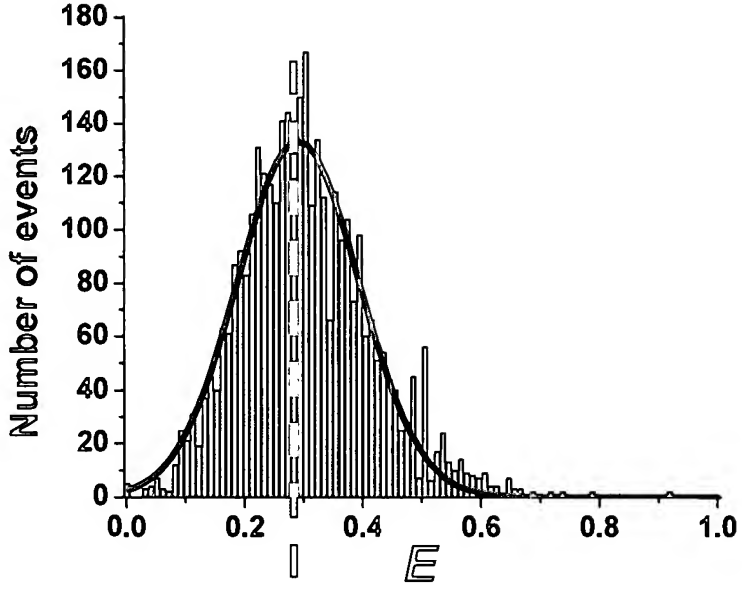




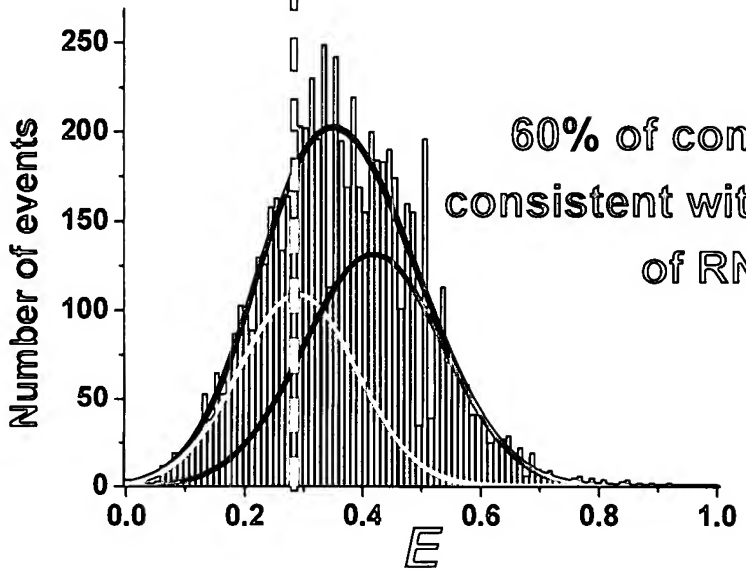
RNAP "chaseability" = 80%

LEADING-EDGE spFRET DETECTS MOVEMENT OF LEADING EDGE DURING ABORTIVE INITIATION

RPo + ApA
($RP_{itc,2}$)



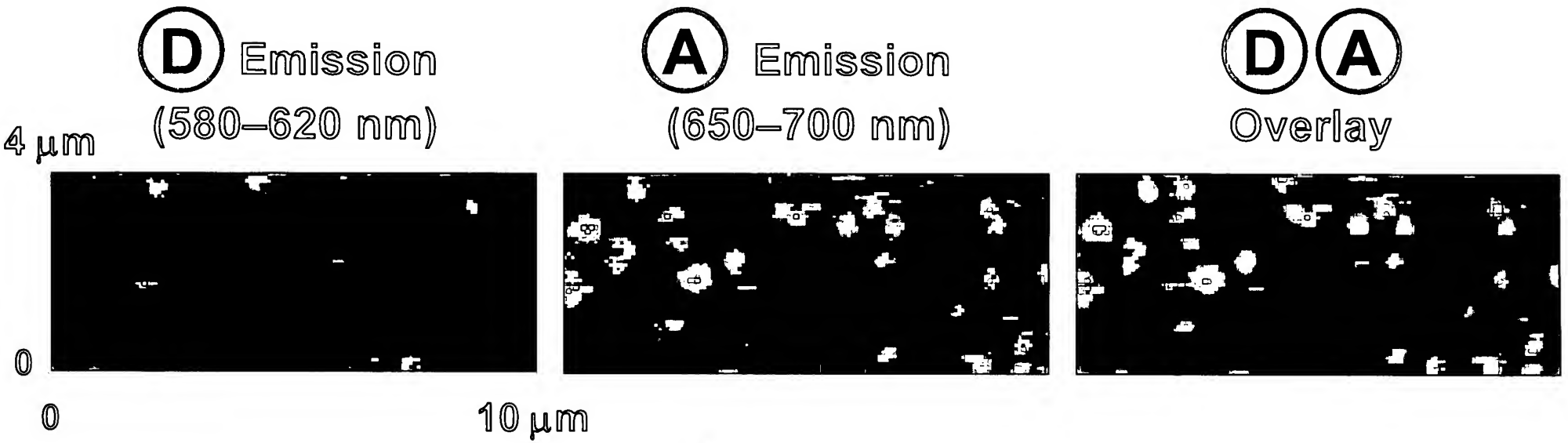
RPo + ApA
+ 25 μM UTP/GTP
($RD_{e,7}$)



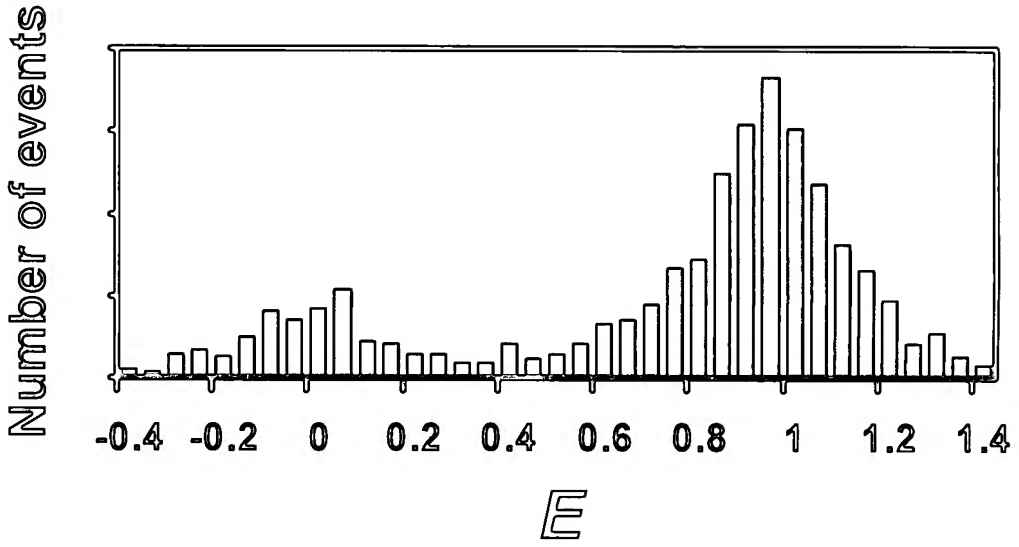
60% of complexes show higher *E*; consistent with downstream movement of RNAP leading edge

TRAILING-EDGE spFRET ON SURFACE-IMMOBILIZED RP_o COMPLEXES

Excitation: 514 nm line of Ar^+ laser

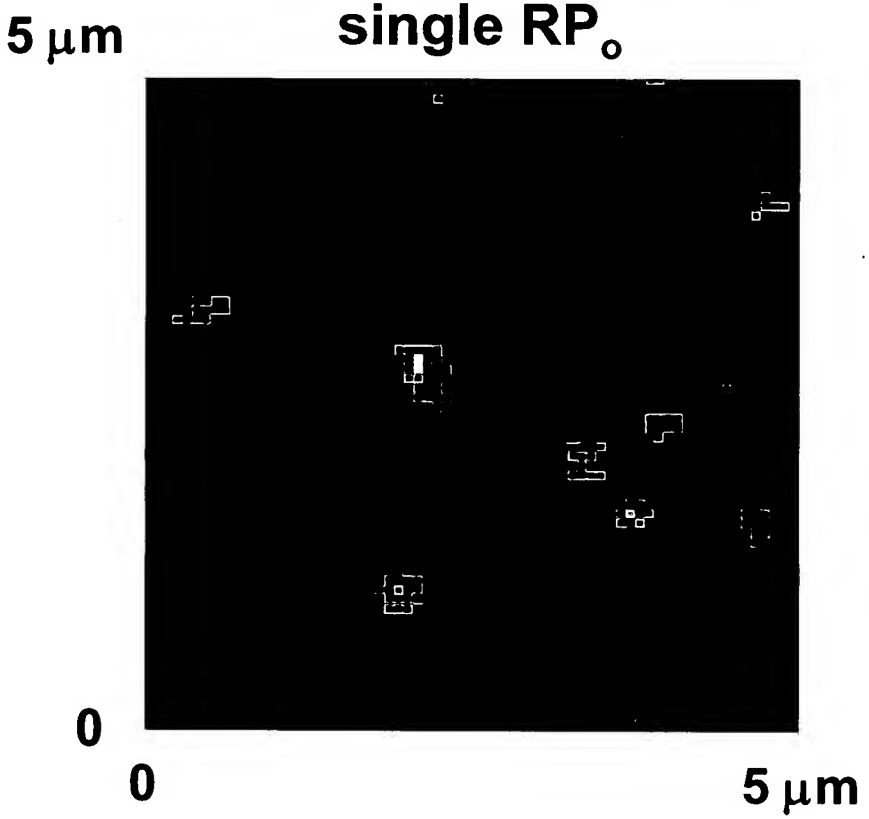


$$E = \frac{I_A}{I_A + \gamma I_D}$$

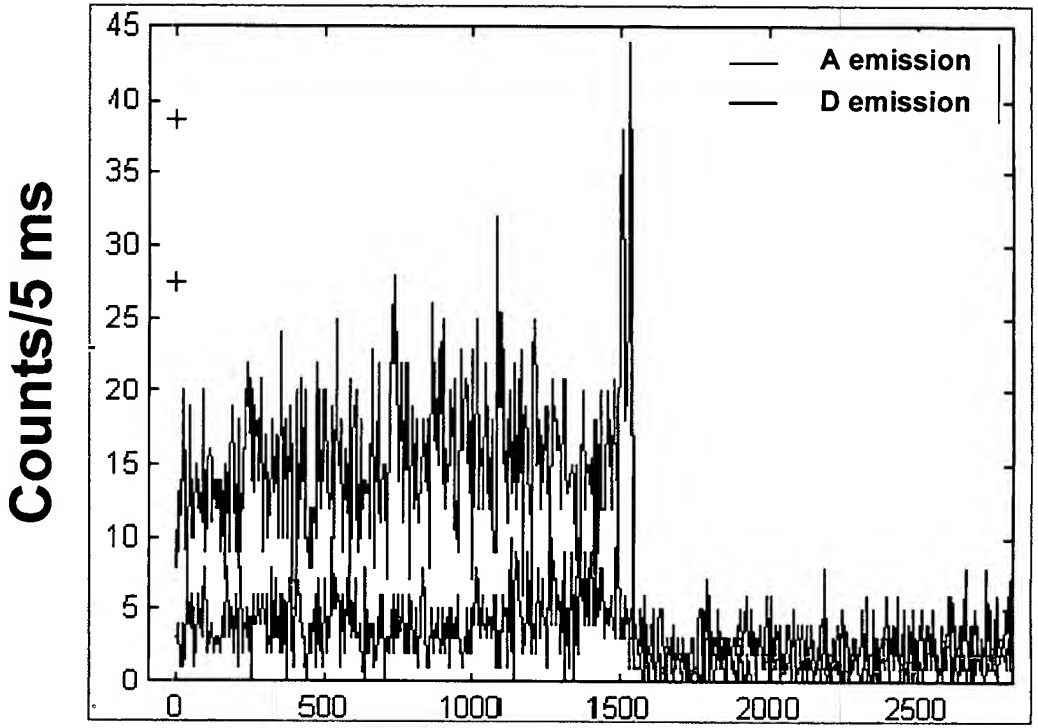


IMAGING AND TIME-TRAJECTORIES OF SINGLE RP_o COMPLEXES

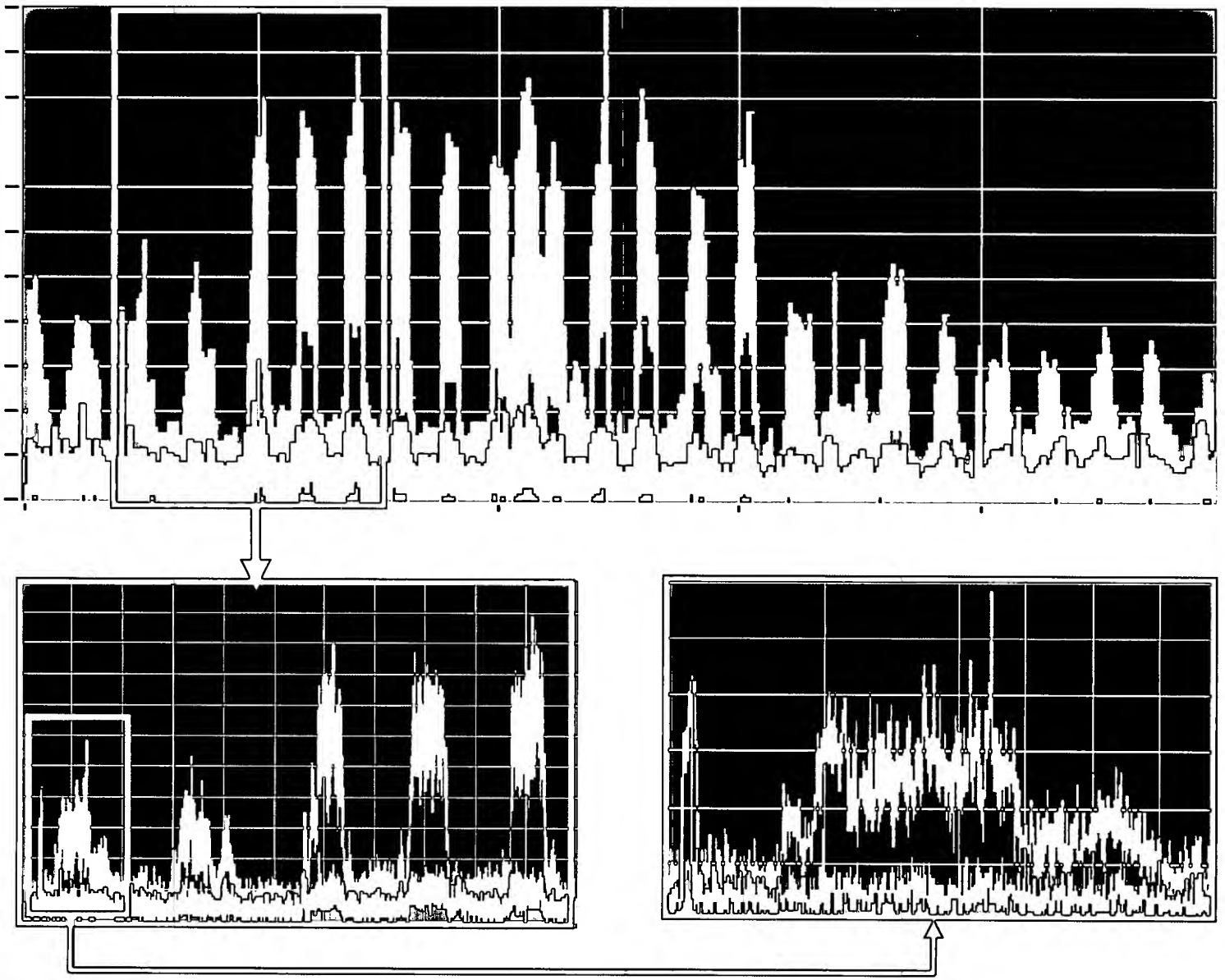
Single-step photobleaching: evidence for imaging single RP_o



Time-trajectory for a single RP_o showing TE-FRET



MONITORING SINGLE-ENZYME DYNAMICS ON IMMOBILIZED MOLECULES



CONCLUSIONS

- Developed robust assays for analysis of structure, dynamics, and activity of protein-DNA complexes
- Confirmed sigma presence in early elongation complexes
- Determined activity for translocation and for chase reactions
- Detected movement of leading edge during abortive initiation
- Future work:
    - Abortive initiation mechanism
    - Sigma dynamics at various transcription steps

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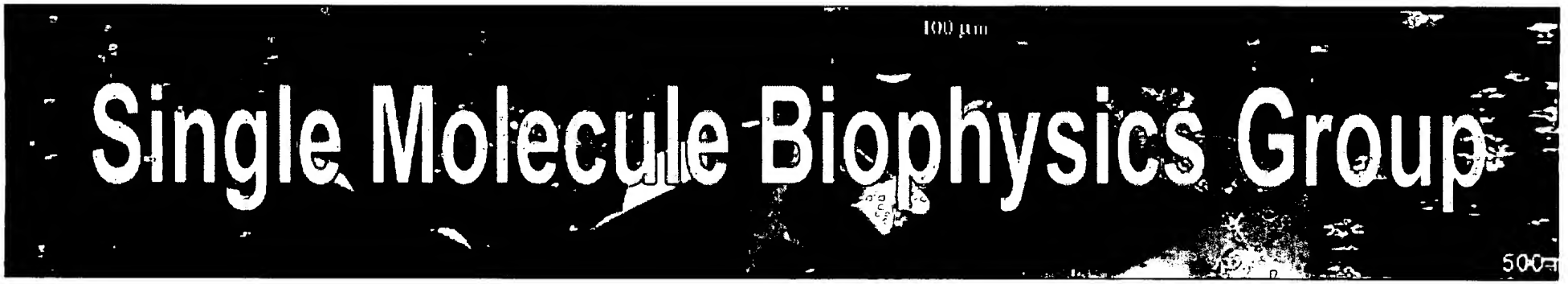
Vladimir Mekler
Jayanta Mukhopadhyay
Andrey Revyakin

Philip Tinnefeld (U.Heidelberg)

and all SMBs!

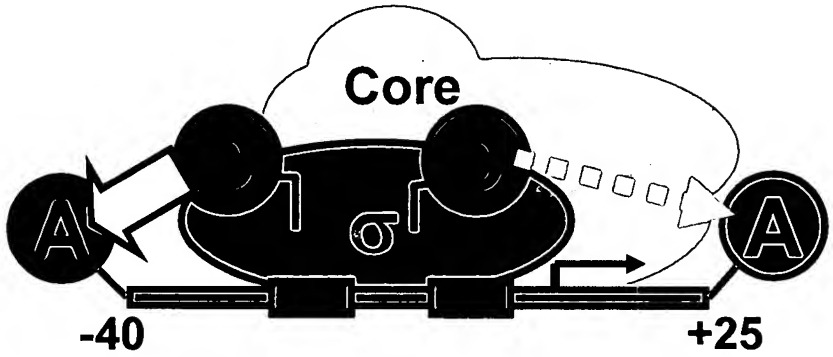


Funding: DOE, NIH

TRAILING-EDGE and LEADING-EDGE FRET:

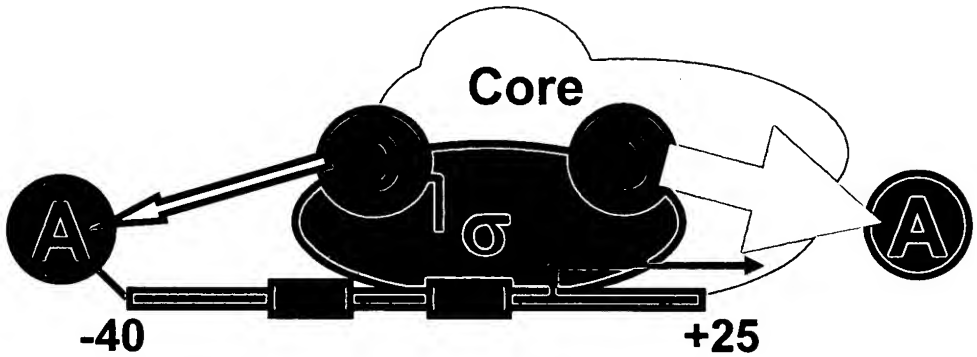
Assay of translocation of a protein relative to a nucleic acid

Trailing-edge/leading-edge FRET (TELE-FRET)



Ruler 1: High FRET

Ruler 2: Low FRET

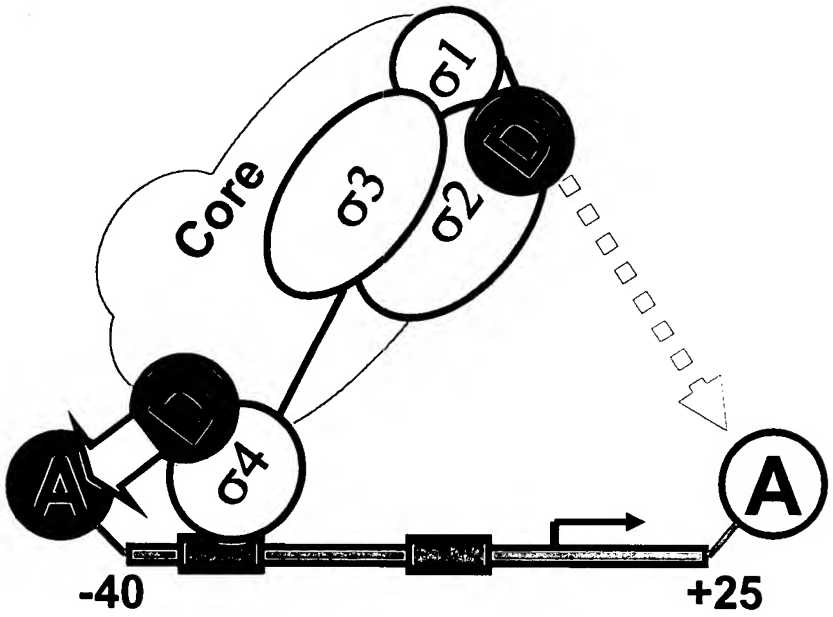


Ruler 1: Low FRET

Ruler 2: High FRET

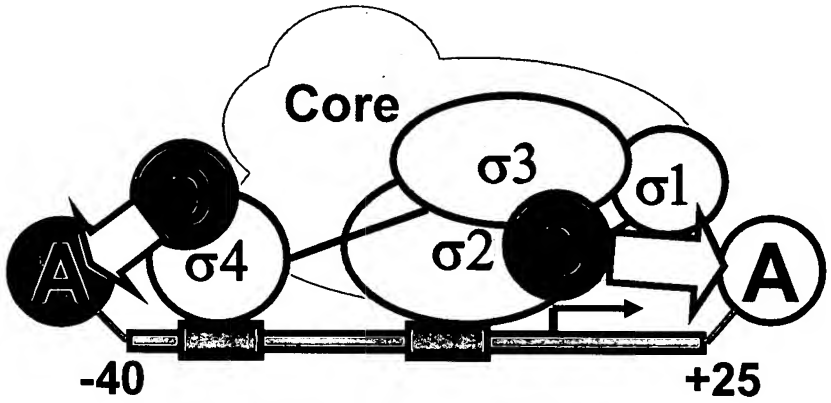
Mukhopadhyay *et al.*, 2001; Mekler *et al.*, 2002

Step-Sequence of formation of promoter contacts using 2 FRET rulers



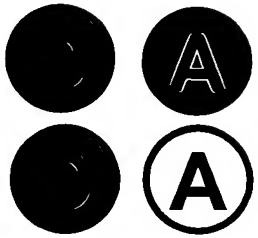
Ruler 1: High FRET

Ruler 2: Low FRET



Ruler 1: High FRET

Ruler 2: High FRET



Ruler 1

Ruler 2